AWARD NUMBER: W81XWH-14-1-0448

TITLE: Characterizing the Hypermutated Subtype of Advanced Prostate Cancer as a Predictive Biomarker for Precision Medicine

PRINCIPAL INVESTIGATOR: Colin Pritchard

CONTRACTING ORGANIZATION: University of Washington
Seattle, WA 98195-7110

REPORT DATE: October 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2017	Annual	09/15/2016 – 09/14/2017

4. TITLE AND SUBTITLE

Characterizing the Hypermutated Subtype of Advanced Prostate Cancer as a Predictive Biomarker for Precision Medicine

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0448

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Pritchard, Colin C.

E-mail: cpritch@uw.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Washington
4333 Brooklyn Ave NE
Seattle, WA 98195-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal of this research is to characterize the mechanisms leading to hypermutated prostate cancer and to integrate tumor hypermutation status with clinical decision making and therapy to improve the care of men with advanced prostate cancer. We identified 10/103 patients (10% of men) with hypermutated advanced prostate cancers. Using a targeted deep sequencing assay that includes intronic and flanking regions we discovered DNA mismatch repair (MMR) gene mutations in all hypermutated tumors. Mutations were commonly complex genomic rearrangements in the *MSH2* and *MSH6* mismatch repair genes. There was loss of the corresponding MMR protein expression in tumor tissue and phenotypic microsatellite instability in every hypermutated tumor. Our results support that microsatellite instability resulting from loss of function mutations in DNA mismatch repair genes is the major mechanism leading to hypermutation in prostate cancer. We have developed the mSINGS method to detect phenotype microsatellite instability from next-generation sequencing data. This method accurately classified hypermutated prostate cancers. We have successfully applied mSINGS to targeted capture assays (and to exome data. We have developed a clinical assay termed MSIplus based on the mSINGS method. We have completed work on PDX models to test responsiveness to specific therapies and have begun to recruit men with prostate cancer in a pilot study to test for MSI and hypermutation. We and others have observed that prostate cancer patients with hypermutated MSI tumors may be responsive to checkpoint blockade immunoterhapy. During the no-cost extension year we plan to focus work on aim 4, which will involve continued testing of MMR gene mutations using UW-OncoPlex and adapting the MSIplus test to optimize MSI sensitivity to identify men with prostate cancer for checkpoint blockage immunotherapy.

15. SUBJECT TERMS
Prostate cancer, hypermutation, hyper-mutation, microsatellite instability, MSI, MLH1, MSH2, MSH6, PMS2, metastasis, precision medicine, immunotherapy

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	213	

Table of Contents

Page

1. **Introduction……4**
2. **Keywords……4**
3. **Accomplishments……..……..5**
4. **Impact……..……26**
5. **Changes/Problems..…..……28**
6. **Products……..……..……28**
7. **Participants & Other Collaborating Organizations……32**
8. **Special Reporting Requirements……34**
9. **Appendices…….……..35**

1. INTRODUCTION

The goal of this project is to characterize the mechanisms leading to hypermutated prostate cancer and to integrate tumor hypermutation status with clinical decision making and therapy to improve the care of men with advanced prostate cancer. Using Next-Gen sequencing approaches my colleagues at the University of Washington recently identified a hypermutated phenotype/genotype in 10-20% of advanced prostate cancers. This phenotype was subsequently observed in primary prostate cancer. Prostate cancer hypermutation is a promising target for precision therapy, but the mechanisms leading to hypermutation, optimal methods to measure hypermutation status in the clinic, and clinical implications for prostate cancer patients are not yet understood. Our hypothesis is that hypermutated advanced prostate cancer is caused by defects in genes regulating DNA repair pathways, which can be accurately identified using existing clinical diagnostics, and that hypermutation status can predict responses to therapy.

2. KEYWORDS

Prostate cancer, hypermutation, hyper-mutation, microsatellite instability, MSI, MLH1, MSH2, MSH6, PMS2, metastasis, precision medicine

3. ACCOMPLISHMENTS

Accomplishments in the first, second, and third years for research-specific tasks are reported according to major goals of the project in the approved SOW, and organized by specific aim.

3.1 What were the major goals of the project?

Specific Aim 1: Identify mechanisms that drive the hypermutated phenotype in advanced prostate cancer.	**Months**	**Completed?**
Major Task: Sequence DNA repair pathway genes in advanced prostate cancer tumor samples	**1-12**	**Yes**
Subtask 1: Examine hypermutated and non-hypermutated UW prostate cancer rapid autopsy samples using BROCA and UW-OncoPlex assays	1-6	**Yes**
Subtask 2: Assess for functional loss of DNA repair pathway gene expression by IHC, and MSI PCR	3-12	**Yes**
Milestone(s) Achieved: identification of specific mutated DNA repair pathway genes in hypermutated prostate cancer	12	**Yes**

Specific Aim 2: Determine unique vulnerabilities of hypermutated prostate cancer to therapy in xenograft models.	**Months**	**Completed?**
Major Task: Assess differential responses to chemotherapy and targeted therapy in LuCaP tumor cell lines xenografted in mice	12-36	Yes
Subtask 1: Use xenograft LuCaP hypermutated prostate cancer cells lines 58, 73, and 147 and 3 non-hypermutated control cell lines. Assess xenograft tumor responses to chemotherapy and targeted therapy agents	12-36	Yes
Milestone(s) Achieved: Identification of differential efficacy of targeted therapies in hypermutated prostate cancer	24-36	Yes

Specific Aim 3: Develop and validate a clinical diagnostic approach to determine hypermutation status in advanced prostate cancer.	**Months**	**Completed?**
Major Task: Establish a clinical assay(s) to detect tumor hypermutation	1-24	Yes
Subtask 1: Develop bioinformatics methods to accurately detect hypermutation and microsatellite instability using the UW-OncoPlex assay	1-12	Yes
Subtask 2: Establish the performance characteristics of MSI-PCR and IHC-based approaches to detect hypermutation compared to the UW-OncoPlex genomic sequencing	12-24	Yes
Milestone(s) Achieved: Clinically validated approach to detect the hypermutated subtype of advanced prostate cancer established	24	Yes
Milestone(s) Achieved: Original manuscript on bioinformatics method on detected MSI by next-generation sequencing	12-24	Yes

Specific Aim 4: Implement diagnostic testing for hypermutation status in the UW-OncoPlex program for precision cancer medicine.	**Months**	**Completed?**
Major Task: Clinical trial of UW-OncoPlex testing in advanced prostate cancer that includes assessment of hypermutation status	24-36	Partially
Subtask 1: Establish a clinical trial that includes hypermutation testing by UW-OncoPlex with or without additional MSI-PCR/MSI-IHC tests depending on results of Aim 3	24-36	Yes
Subtask 2: Report hypermutation status results to medical oncologists in prostate cancer precision tumor board meetings and document treatment decisions and short-term outcomes.	24-36	Partially
Milestone(s) Achieved: Hypermutation status is used in clinical decision making for men with advanced prostate cancer with feedback on outcomes	36	Partially
Milestone(s) Achieved: Manuscript describing the clinical role of tumor hypermutation status as a predictive biomarker for advanced prostate cancer	36	Partially

3.12 What was accomplished under these goals?

Specific Aim 1: Identify mechanisms that drive the hypermutated phenotype in advanced prostate cancer

Work on Specific Aim 1 was largely completed in Year 1 and 2 and is summarized below. We published a manuscript in *Nature Communications* based on the work accomplished in Aim 1 (Pritchard et al. *Nat Commun.* 2014 5:4988, see Appendix 1). In Year 2 we completed sequencing of all available patient samples from the UW rapid autopsy cohort.

Specific Aim 1, Subtask 1: *Examine hypermutated and non-hypermutated UW prostate cancer rapid autopsy samples using BROCA and UW-OncoPlex assays*

We hypothesized that mutations in key DNA repair pathway genes lead to the hypermutated subtype of advanced prostate cancer, most likely mutations in DNA mismatch repair genes. To test this hypothesis we performed targeted deep sequencing of DNA repair genes in hypermutated and non-hypermutated advanced prostate cancer samples from two sources: LuCaP xenograft lines and tumors from the UWMC rapid autopsy program. Both tumor sources consisted primarily of castration resistant prostate cancer (CRPC). Using exome sequencing we identified 3 hypermutated patient-derived xenograft (PDX) lines (LuCaP 58, LuCaP 73, and LuCaP 147) and 8 of 91 rapid autopsy patients with hypermutated tumors (05-165, 03-130, 06-134, 00-010, 05-123, 01-002, 04-108, 99-111). There was partial overlap between the PDX and the autopsy cases because some LuCaP lines had been derived from the autopsy patients. There were a total of 10 out of 103 unique patients who had hypermutated tumors, for an overall prevalence of 9.7% in our cohort.

We performed the BROCA targeted DNA capture and massively parallel sequencing assay that assesses single nucleotide variants (SNVs), small insertions and deletions (indels), copy number variants (CNVs), and structural variants (SVs) in DNA repair genes simultaneously. Importantly, the BROCA assay includes capture of complete genes including introns and flanking sequences, which is in contract to exome sequencing which captures exons only. This detail proved to be crucial to our success in this research aim. We sequenced samples to an average of ~800x depth, multiplexing 24 samples per lane on a HiSeq2500. The BROCA assay uses the Agilent SureSelect enrichment system to capture the coding exons and flanking splice sites of genes listed in **Table 1**.

Table 1: BROCA genes (assay version 6)

DNA Repair Pathways	*ATM*	*ATR*	*BAP1*	*BARD1*	*BRCA1*	*BRCA2*	*BRCC3*	*BRIP1*
	CHEK1	*CHEK2*	*FAM175A*	***MLH1***	*MRE11A*	***MSH2***	***MSH6***	*NBN*
	PALB2	***PMS2***	*PRSS1*	*PTEN*	*RAD50*	*RAD51B*	*RAD51C*	*RAD51D*
	RBBP8	*TP53*	*TP53BP1*	*XRCC2*				
Additional Cancer-Related	*AKT1*	*APC*	*BMPR1A*	*CDH1*	*CDK4*	*CDKN2A*	*CTNNA1*	*GALNT12*
	GEN1	*GREM1*	*HOXB13*	*MEN1*	*MUTYH*	*PIK3CA*	***POLD1***	***POLE***
	PPM1D	*RET*	*SDHB*	*SDHC*	*SDHD*	*SMAD4*	*STK11*	*VHL*

To assess within-patient tumor mutation heterogeneity we tested up to 4 different metastatic sites in a subset of patients. For each patient we also tested matched normal (non-tumor) tissue to determine if mutations were inherited or somatic.

All three PDX hypermutated tumors had complex structural rearrangements in *MSH2*, *MSH6* or both genes (**Table 2**), while only 1 of 20 non-hypermutated xenografts had mutations in these genes (LuCaP 145, derived from a patient with neuroendocrine prostate cancer, Supplementary Fig. 4). A second loss-of-function mutation in *MSH2* or *MSH6* was detected in the three hypermutated PDX tumors, but not in LuCaP 145, supporting a requirement for bi-allelic gene inactivation underlying the hypermutated genome.

We performed the BROCA targeted DNA capture and massively parallel sequencing assays that assesses single nucleotide variants (SNVs), small insertions and deletions (indels), copy number variants (CNVs), and structural variants (SVs) in DNA repair genes simultaneously.

All three PDX hypermutated tumors had complex structural rearrangements in *MSH2*, *MSH6* or both genes (**Table 2**), while only 1 of 20 non-hypermutated xenografts had mutations in these genes (LuCaP 145, derived from a patient with neuroendocrine prostate cancer). A second loss-of-function mutation in *MSH2* or *MSH6* was detected in the three hypermutated PDX tumors, but not in LuCaP 145, supporting a requirement for bi-allelic gene inactivation underlying the hypermutated genome.

Table 2: Mismatch Repair (MMR) Gene Mutations Detected in All Hypermutated Prostate Cancers

Patient**	Hypermutated	MSI	MMR Gene Mutation(s)*
05-165 (LuCaP 147)	Yes	Yes	MSH2-C2orf61 inversion, MSH2-KCNK12 inversion
00-010	Yes	Yes	MSH2 frameshift
05-123	Yes	Yes	MSH2 frameshift
03-130	Yes	Yes	MSH2 translocation t(2;18)
06-134	Yes	Yes	MLH1 homozygous copy loss
LuCaP 58	Yes	Yes	MSH6 del exon 8 to 3'UTR, MSH6 frameshift
LuCaP 73	Yes	Yes	MSH6 3Mb inversion + frameshift, MSH2 440kb inversion
01-002	Yes	Yes	MSH2 exon 1-8 del (germline with LOH)
04-108	Yes	Yes	MSH2 rearrangement
99-111	Yes	Yes	MSH2 exon 1-8 del (no matched germline)

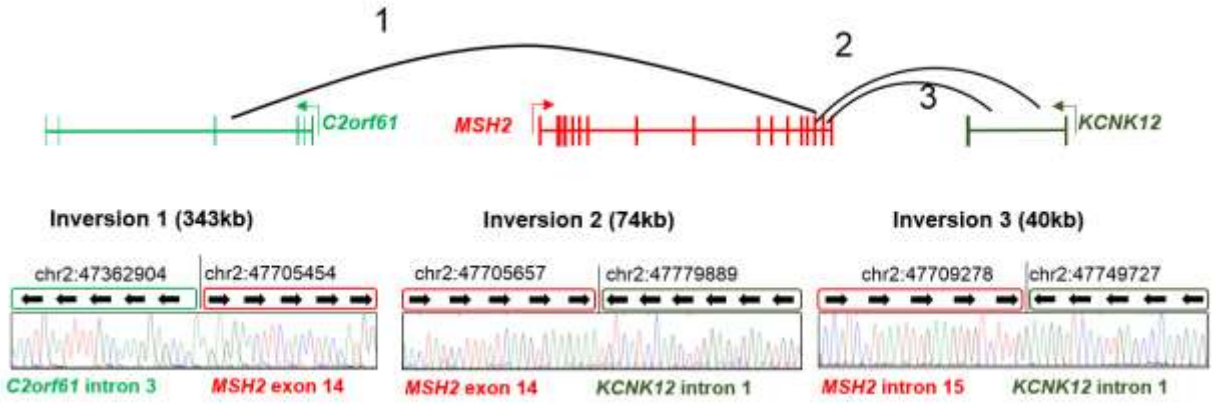
*Mosaic *MSH6* frameshift mutations observed in a poly G tract in exon 5 (c.3261dup/del) and poly A tract in exon 7 (c.3573del) were detected in several hypermutated samples and are not included in the table because they are presumed to be due to MSI.
**LuCaP 147 is derived from patient 05-165

We detected mutations with predicted loss-of-function in *MSH2*, *MSH6*, or both genes in 7 of 8 rapid autopsy patients with hypermutated tumors. Mutations included complex structural

rearrangements, copy losses, and frameshift mutations (**Table 2**). One hypermutated patient had mutations in the MMR gene *MLH1*. In all patients where multiple sites were tested hypermutation status and MMR mutations were concordant at different metastatic sites tested in the same patient. MMR mutations were somatic except for patient 01-002, who had a germline *MSH2* deletion and Lynch syndrome.

.

A) *MSH2* Structural Rearrangement in Hypermutated Autopsy Tumor 05-165 and LuCaP 147



B) *MSH2* Structural Rearrangement in Hypermutated Autopsy Tumor 03-130

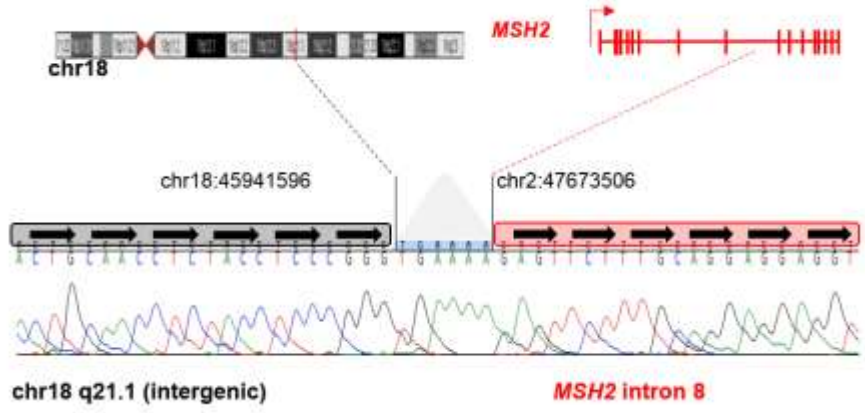


Figure 1: Examples of complex *MSH2* structural rearrangement detected in hypermutated prostate tumors. A) In autopsy sample 05-165 and patient-derived xenograft LuCaP 147 is a representative complex *MSH2* rearrangement (LuCaP 147 was derived from autopsy patient 05-165). B) *MSH2* structural rearrangement in hypermutated autopsy tumor 03-130. Breakpoints were confirmed by Sanger sequencing. Genomic coordinates are build hg19. A total of 4 or 7 hypermutated cases had complex rearrangements in *MSH2* and *MSH6* or both genes.

To cross-validate mutation calling for our UW-OncoPlex targeted sequencing platform that is the focus of clinical sequencing work for precision medicine we tested one hypermutated rapid autopsy prostate cancer case (00-010) and two non-hypermutated autopsy cases (00-029 and 00-090) using UW-OncoPlex. We also tested one LuCaP line (LuCaP 23.1). Among the genes that overlap the two panels there was 100% concordance of somatic coding mutation calls that were present at >5% variant allele fraction between the two platforms.

Specific Aim 1, Subtask 2: *Assess for functional loss of DNA repair pathway gene expression by IHC, and MSI PCR*

MSH2 and *MSH6* are mismatch DNA repair genes that act together as a heterodimer, and bi-allelic inactivating mutations of either gene are predicted to result in microsatellite instability (MSI). PCR of microsatellite loci revealed MSI in all hypermutated tumors, from both PDX and autopsy patients (**Figure 2**, **Table 2**). IHC for DNA mismatch repair proteins in hypermutated tumors demonstrated complete loss of MSH2 and/or MSH6 in a pattern consistent with the inactivating mutations detected by sequencing (**Figure 3**). Non-hypermutated tumors were microsatellite stable and had intact MSH2 and MSH6 protein.

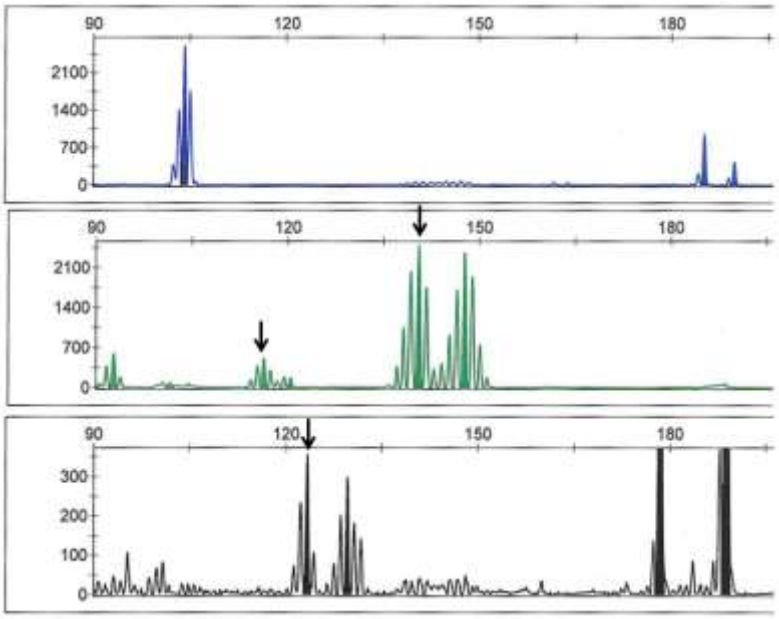


Figure 2: Hypermutated tumors are MSI-High. Hypermutated tumors exhibited microsatellite instability by PCR. Shown is representative data for LuCaP 58 which is positive for MSI in 3/5 mononucleotide marker systems (BAT25, MONO27, NR26, arrows). All hypermutated tumors were MSI-PCR positive in at least 2/5 loci.

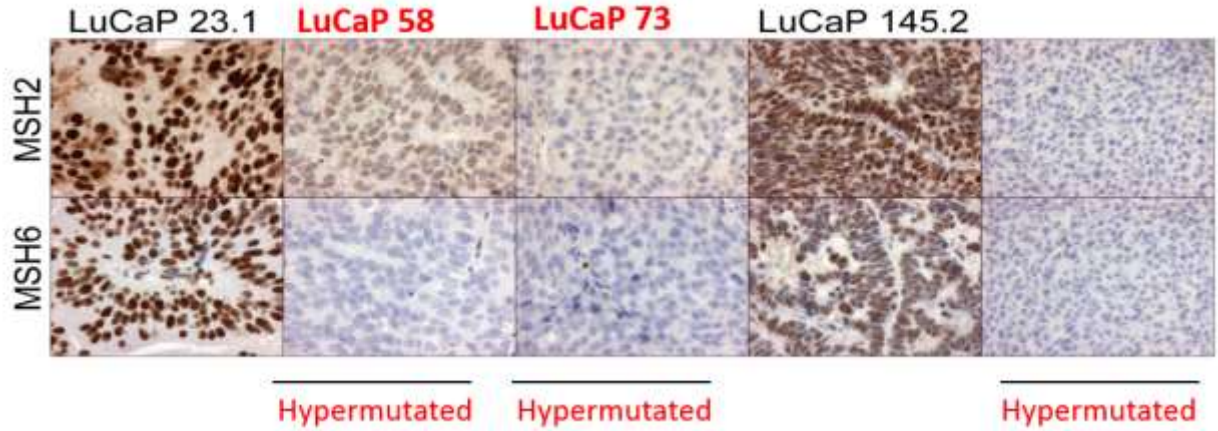


Figure 3: Hypermutated have loss of MSH2 and MSH6 protein by IHC. Similar results were observed in hypermutated tumors from rapid autopsy patients (see Appendix 1).

The findings support the conclusion that the hypermutated subtype of prostate cancer is chiefly due to loss-of-function mutations in *MSH2* and *MSH6* that result in MSI. Most interestingly, 6 of 10 hypermutated cases had complex structural rearrangements in *MSH2* and *MSH6* that were not detected by exome sequencing in the same samples, and would also not be expected to be detected by traditional exon-based Sanger sequencing methods. Previous studies have reported MMR protein loss and MSI in both primary and advanced prostate cancers, but very few MMR mutations have been identified. We speculate that technical limitations have led to an underestimation of MMR gene mutations in prostate cancer.

Specific Aim 2: Determine unique vulnerabilities of hypermutated prostate cancer to therapy in xenograft models.

Aim 2, Subtask 1: *Use xenograft LuCaP hypermutated prostate cancer cells lines 58, 73, and 147 and 3 non-hypermutated control cell lines. Assess xenograft tumor responses to chemotherapy and targeted therapy agents*

Work on aim 2 was started in year 2 and completed in year 3. Note that no funding for animal studies was provided by this award. The goal of this aim is to carry out a pilot study using patient-derived xenograft (PDX) preclinical models as 'tumor avatars' to test anti-cancer therapies in comparison to responses in non-hypermutated LuCaP xenograft lines. We are assessing responses to currently used and approved chemotherapeutics including docetaxel, carboplatin and 5-fluorouricil, to determine if the hypermutated subtype is more or less susceptible to drugs that can be immediately used in clinical practice. We have identified 3 hypermutated PDX lines (LuCaP 58, 73, and 147) that we are using to assess selective responses of these therapies, in collaboration with Drs. Colm Morrissey, Robert Vessella, and Eva Corey at the University of Washington GU Cancer Research Laboratory.

For hypermutated PDX tumor LuCaP 147 we obtained 4 different metastatic sites from the patient from whom the xenograft line was derived and found that the same complex *MSH2* structural rearrangements were present in all metastatic sites in the pre-xenografted tumors, demonstrating that the MMR gene structural rearrangements are not an artifact of xenografting.

In collaboration with Drs. Corey, Vessella, Morrissey, and Nelson, we evaluated the efficacy of docetaxel, carboplatin, and 5-FU in hypermutated LuCaP xenografts (LuCaP 58, 73 and 147) and non–hypermutated LuCaP xenografts (LuCaP 70, 96CR, 141, 70, 35, and 35CR). LuCaP tumors were subcutaneously implanted into SCID male mice. When tumor exceeded 100mm^3, animals were randomized and enrolled into following groups: 1) Docetaxel treatment at 10 mg/kg, 2) Docetaxel treatment at 20 mg/kg, 3) Carboplatin at 50 mg/kg twice weekly, 4) 5-FU at 50mg/kg qweek, 5) Vehicle controls/no treatment animals, and 6) Castration animals. Tumor volumes and body weight were measured once weekly.

Treatment responses for the two different docetaxel dosages (10 mg/kg and 20 mg/kg) varied across the hypermutated and non-hypermutated tumor models. For hypermutated models, 10 mg/kg treatment responses ranged from major tumor growth inhibition in LuCaP 58 to mostly unimpeded tumor progression in LuCaP 147. At 20 mg/kg, LuCaP 73 exhibited maximal

responsiveness as opposed to LuCaP 58 and 147 (**Figure 4**). We also assessed single animal response to docetaxel (20 mg/kg) within the hypermutated LuCaP PDX and found heterogeneity in responses. Similar to hypermutated LuCaP PDX, the non–hypermutated LuCaP PDX exhibited a broad range of susceptibility to docetaxel. Comparing the responses of hypermutated and non–hypermutated LuCaP PDX, we did not find a significant difference in susceptibility to docetaxel, or a differential survival benefit. Our results suggest a range of docetaxel responsiveness among LuCaP PDX lines that is not strongly predicted by hypermutation status.

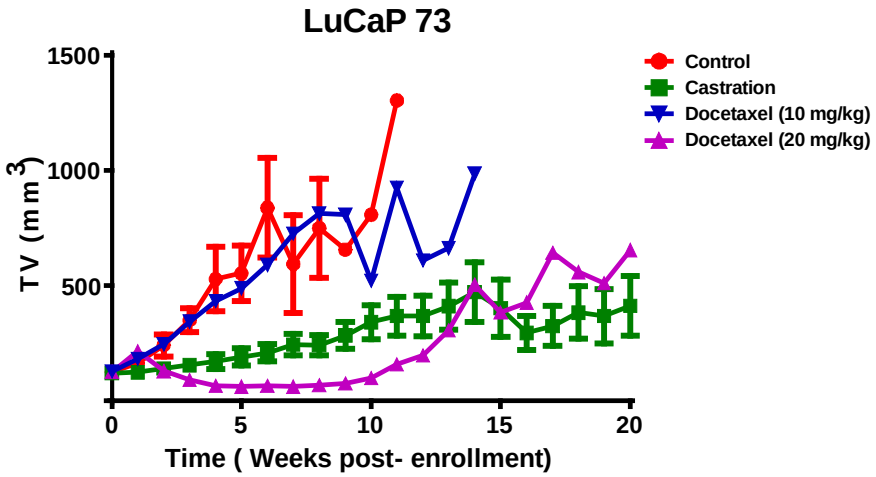


Figure 4. Responses to docetaxel in hypermutated prostate cancer PDX LuCaP 73.

In year 3 we focused on carboplatin and 5-FU and completed similar experiments in the hypermutated and non-hypermuated LuCaP lines using carboplatin and 5-FU. 5-FU at 50mg/kg did not have a significant impact on tumor inhibition in either hypermutated or non-hypermutated lines. Carboplatin at 50mg/kg twice weekly dosing was high effective in halting tumor growth in hypermutated lines **(Figure 5**). However, similar carboplatin responses were also observed for the non-hypermutated lines (**Figure 6**). Because recent data supports that carboplatin is expected to be effective in PDX with homologous recombination DNA repair deficiency, a subgroup analysis of the non-hypermutated lines is planned to evaluate and compare responses in lines with or without HR DNA repair deficiency.

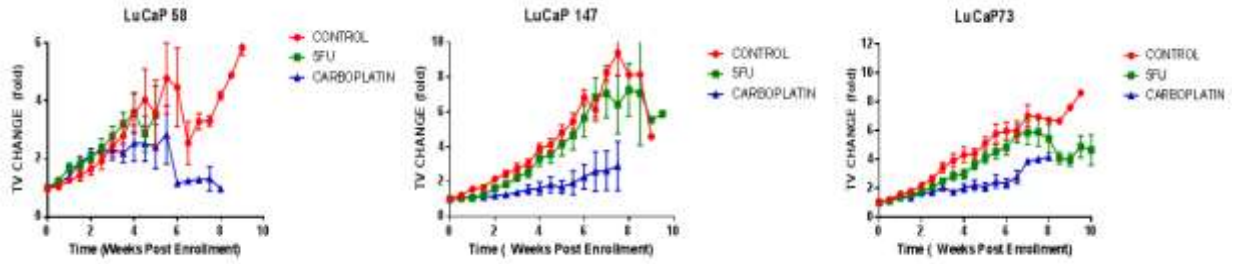
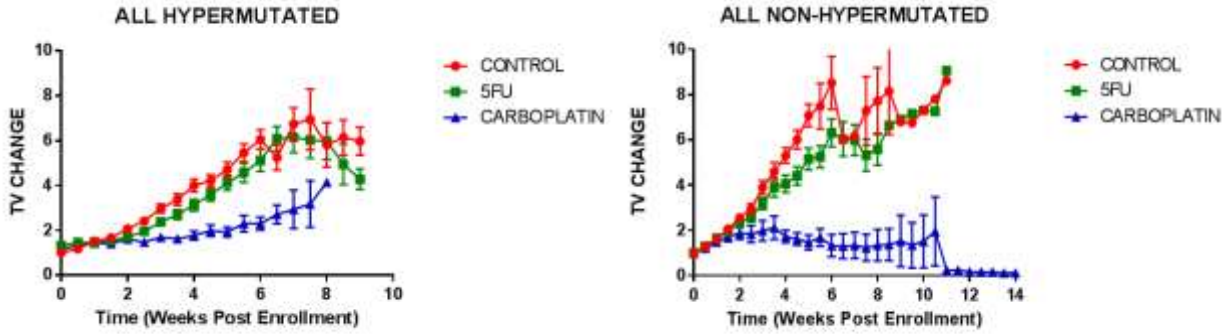


Figure 5: Carboplatin and 5-FU responses in hypermutated LuCaP PDX lines.

Figure 6: Carboplatin and 5-FU responses in hypermutated and non-hypermuated LuCaP PDX lines.



Specific Aim 3: Develop and validate a clinical diagnostic approach to determine hypermutation status in advanced prostate cancer.

Aim 3, Subtask 1: *Develop bioinformatics methods to accurately detect hypermutation and microsatellite instability using the UW-OncoPlex assay*

Work on Aim 3, has been done in Years 1, 2, and 3. We developed a novel method for inferring MSI and hypermutation from next-generation sequencing data that we call "mSINGS". We recently published a manuscript on this method for which Dr. Pritchard was the senior and corresponding author (Salipante et al. *Clin Chem.* 2014 60:1192-9). A graphical depiction of how the mSINGS method works is given in **Figure 7**.

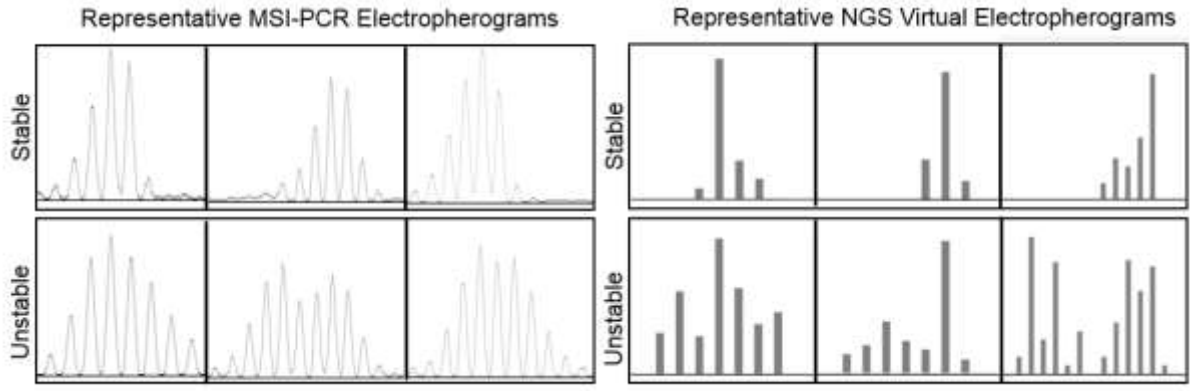


Figure 7: Detection of microsatellite instability by MSI-PCR and next-generation DNA sequencing using "mSINGS". Representative capillary electrophoresis results from MSI-PCR (top panels) and "virtual electropherograms" of next-generation DNA sequencing data (bottom panels), where the length (x-axis) and relative abundance (Y-axis) of variant repeats are plotted. Loci in top and bottom panels are not equivalent, and are from different genomic locations.

We have adapted the mSINGS method to both the BROCA and UW-OncoPlex genomic deep sequencing platforms to accurately detect both phenotypic MSI and hypermutation status, even

when matched non-tumor tissue is not available (**Figure 8**). UW-OncoPlex is a clinically-validated diagnostic platform for precision cancer medicine developed by Dr. Pritchard that has been used to test over 1,000 cancer patients to date (for details on the assay see http://tests.labmed.washington.edu/UW-OncoPlex, or Google: "UW-OncoPlex"). We have identified 65 mononucleotide microsatellite loci that are captured in the current UW-OncoPlex assay version (version 4). We established parameters for each locus to be called unstable based on the SD of peak distribution and defined MSI-High as having at 20% unstable loci. Using the mSINGS informatics approach, we correctly identified all known MSI-High cancer samples (7/7) including one hypermutated prostate cancer sample which had 24/65 (37%) loci unstable (autopsy sample 00-010, liver metastasis). MSI-High samples had 35 +/- 12 unstable loci (n=7, mean +/- SD), while known microsatellite stable samples had only 2 +/- 1.5 unstable loci (n=10).

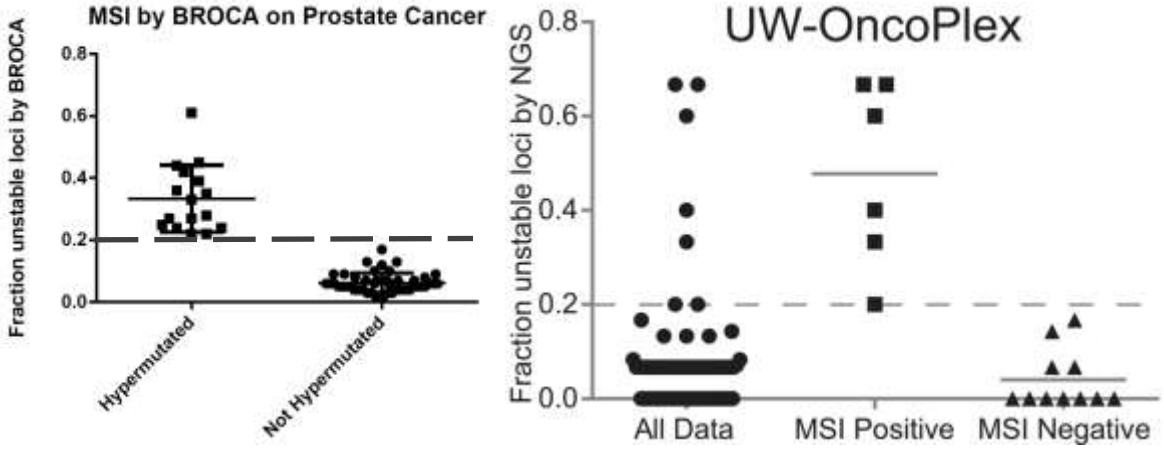


Figure 8: Detection of MSI in prostate cancer samples using mSINGS applied to BROCA and UW-OncoPlex targeted gene sequencing panels. (Left panel) The fraction of unstable microsatellite loci are shown for BROCA (left) and UW-OncoPlex (right) targeted sequencing. Results are stratified by hypermutation or MSI status. The threshold used for interpreting MSI status is indicated by a dashed line, set at a fraction of 0.2 (20% unstable loci). This threshold perfectly separated hypermutated (MSI positive) and not hypermutated (MSI negative) tumors.

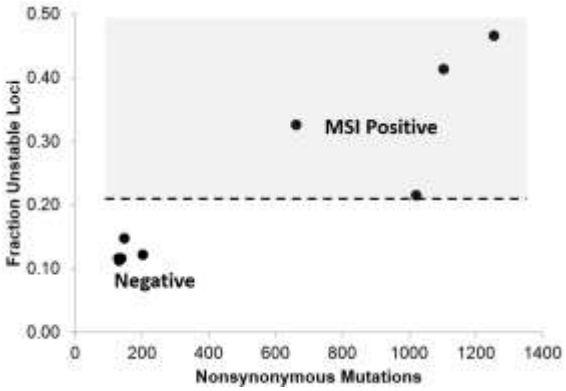


Figure 9. Hypermutated CRPC cases from SU2C international dream team have phenotypic microsatellite instability (MSI) detected by mSINGS. We applied an approach to measure microsatellite instability directly from next-generation sequencing data (mSINGS) to four hypermutated cases, defined as >300 nonsynonymous mutations in exome sequencing. A threshold fraction of 0.2 (20%) unstable loci is the cutoff for microsatellite instability using the mSINGS method (dashed line). All four hypermutated cases were MSI positive and had somatic mutations in mismatch repair genes (*MLH1, MSH2*, or *MSH6*). Selected cases with less than 300 nonsynonymous mutations were MSI negative (bottom).

In collaboration with Stephen Salipante and Jay Shendure we applied the mSINGS method to exome data from 18 available TCGA datasets comprising a total of 5,930 cancer samples. This work led to important mechanistic insights of MSI and was published in *Nature Medicine* (Hause et al. 2016, see Appendix).

We next developed a simple and rapid clinical diagnostic assay based on the mSINGS method that we call "MSIplus". This method using amplicon sequencing of 18 microsatellite loci, following by NGS. We validated this method on 81 tumor specimens with known MSI status, including prostate cancer samples (**Figure 10**). This work has led to a manuscript which is was published in the *Journal of Molecular Diagnostics* (see Appendix). In collaboration with Dr. Michael Schweizer, another CDMRP Physician Scientist Training Award recipient, the MSIplus assay will be used to prescreen patients in a clinical trial of the PD-L1 inhibitor durvalumab in the no-cost extension year.

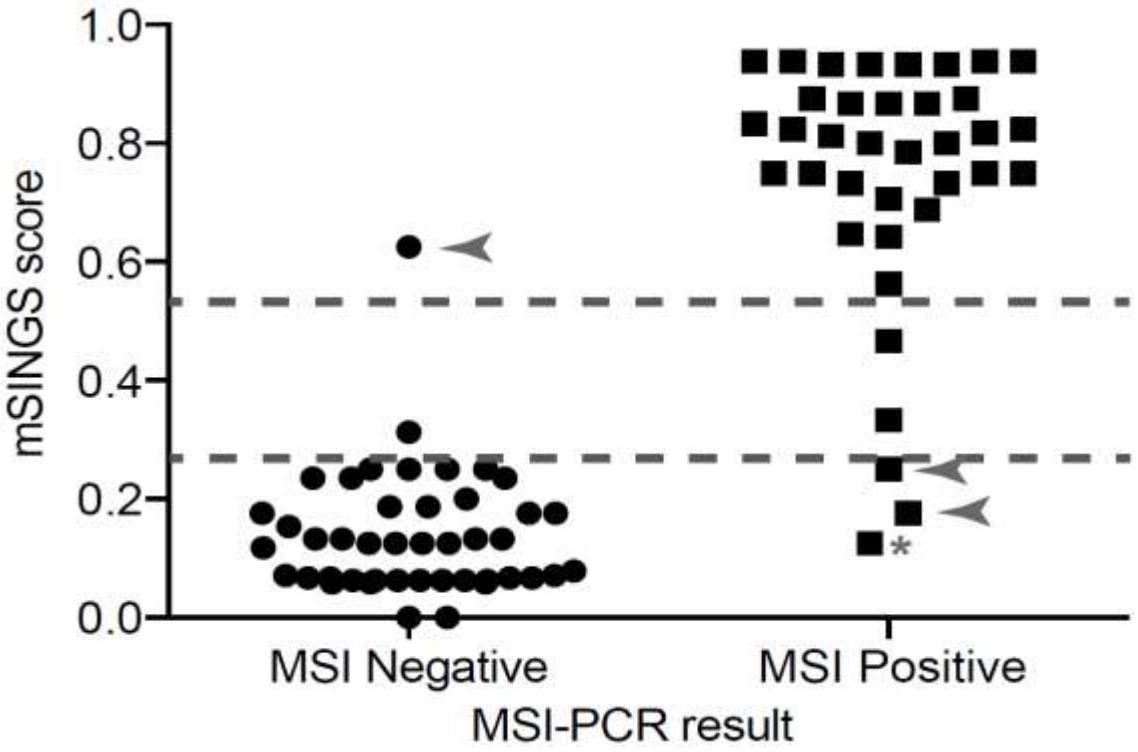


Figure 10: "MSIplus": A rapid and cost-effective stand-alone 18-loci MSI test based on the mSINGS method. 81 tumor samples with known MSI status. Arrows represent potentially discrepant cases in which the original MSI result used as the gold standard was likely to be in error.

Aim 3, Subtask 2: *Establish the performance characteristics of MSI-PCR and IHC-based approaches to detect hypermutation compared to the UW-OncoPlex genomic sequencing*

In Year 3 we applied the MSIplus assay to prostate samples with known mismatch repair deficiency (MMRd) "gold-standard" status, defined by bi-allelic MMR mutation and

corresponding tumor hypermutation detected by the UW-OncoPlex assay. We identified 28 MMRd prostate tumors and 54 MMR-intact prostate tumors and ran these samples on the MSIplus assay and in parallel on the traditional 5-marker Promega capillary electrophoresis assay. MSIplus correctly identified 25/ 28 MMRd cases as MSI-high (89% sensitivity, **Figure 11**) compared to only 20/28 cases identified as MSI-high by the traditional Promega assay (71% sensitivity). All 54 MMR-intact prostate cancer tumors were classified as microsatellite stable by both MSIplus and the Promega assay (100% specificity for both assays).

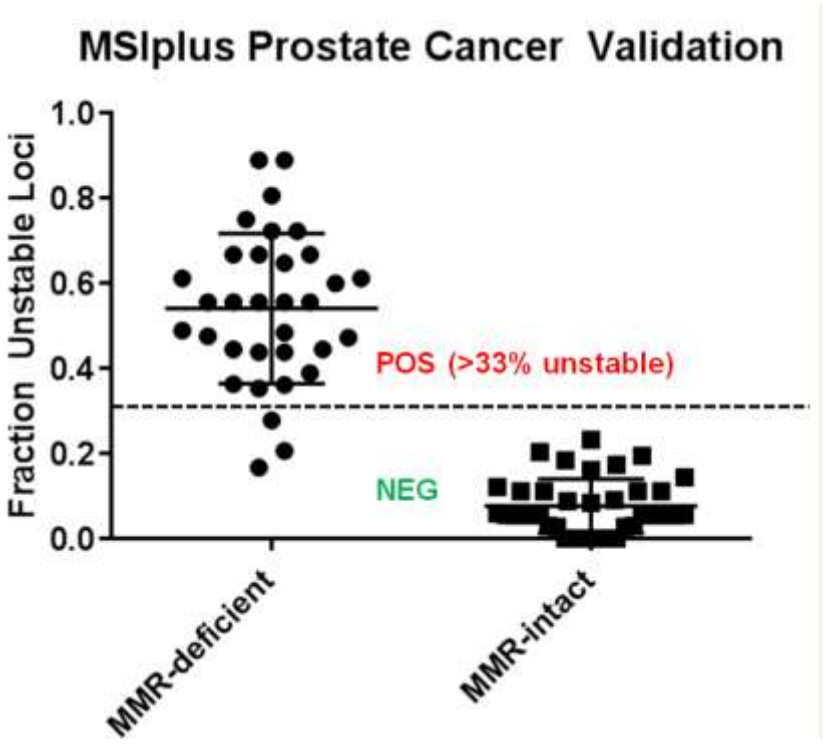


Figure 11: Validation of MSIplus in prostate cancer samples with known "gold-standard" MMRd status.

Through our study of the landscape of MSI in cancer (Hause et al. 2016 *Nature Medicine*) we have discovered several additional microsatellite loci that perform especially well for discriminating MSI in prostate cancer. In collaboration with Dr. Stephen Salipante we plan to adapt the MSIplus assay to evaluate these loci for prostate cancer samples. In the no-cost extension year we plan to add these additional loci to expand the MSIplus panel, and will perform a re-validation study in the hope of further improving the sensitivity of the assay for prostate cancer. We hope the fast and inexpensive MSIplus assay may be able to qualify more prostate cancer patients for checkpoint blockade immunotherapy that has recently been FDA approved for any MSI high tumor type.

With help from mentor Dr. Larry True, we have identified a histologic correlate to hypermutation and MSI status. We found that hypermutated prostate cancer was associated with ductal histology, a rare histologic variant. Ductal prostatic adenocarcinomas (dPC) are an aggressive histopathologic variant of prostate cancer, characterized by large glands lined by tall, pseudostratified, columnar neoplastic epithelial cells (**Figure 12**). Approximately 3% of all

prostate cancers have at least a component of ductal histology, with only 0.2% having pure ductal histology. Clinically, dPCs tend to have a more aggressive course – behaving similarly to Gleason 4+4=8 carcinomas. Tumors with >10% ductal component are associated with a higher stage, are more likely to present with metastatic disease, and may be less responsive to androgen deprivation.

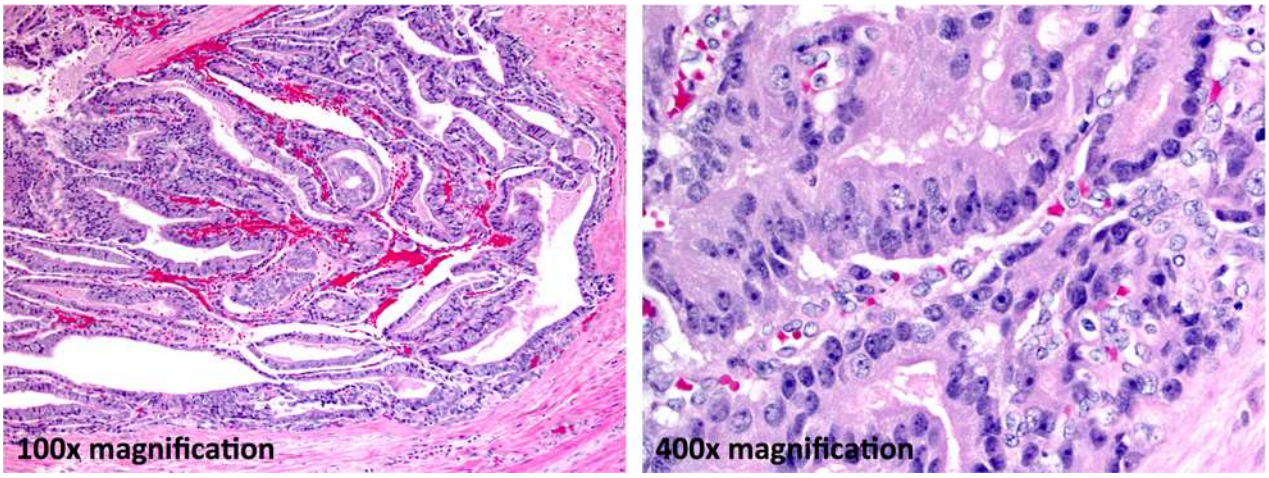


Figure 12: Ductal adenocarcinoma component of a hypermutated prostate cancer case. In this case, approximately 65% of the carcinoma is ductal. Large tumor cell aggregates have a tubulopapillary architecture (100x final magnification). Forming a pseudostratified columnar epithelium the tumor cells have markedly atypical nuclei with clumped chromatin and prominent nucleoli (400x final magnification).

We applied UW-OncoPlex targeted deep sequencing to tumors from 10 consecutive patients with known dPC. Nine of 10 samples had sufficient material for tumor sequencing. Four (40%) patients' tumors had a mismatch repair (MMR) gene alteration (N=2, *MSH2*; N=1, *MSH6*; and N=1, *MLH1*), of which 3 (75%) had evidence of MSI using mSINGS and hypermutation **(Table 3).** The three hypermutated cases with MSI had clear evidence of bi-allelic MMR mutation, while the one case without MSI or hypermutated had only on MMR mutation (subject 2). Similar to what we observed in the rapid autopsy series, MMR mutations were structural rearrangements in 3 of 4 cases. Sections of the primary carcinomas from two of the rapid autopsy patients were available of review; remarkable—both of these tumors had dPC. MMR mutations associated with hypermutation were common in our cohort of dPC patients. The presence of dPC may be a rapid means to enrich populations for further screening for hypermutation and MSI. Given our small sample size, we plan to evaluate additional dPC cases. This work has led to a manuscript that is currently in review.

Table 3: Summary of DNA repair mutations identified in ductal prostate cancer cases.

Subject number	Ductal component of sample used for NGS	MMR gene alteration	HR gene alteration	MSI by mSINGS	Hyper-mutated	Total Coding Mutations (per 1.2Mb sequenced)
1	71%	No	*CHEK2* c.1100delC +LOH	No	No	4
2	**45%**	***MSH2* inversion**	**No**	**No**	**No**	**4**
3	65%	No	No	No	No	4
4	**30%**	***MSH6* c.1900_1901del+LOH**	**No**	**Yes (low)**	**Yes**	**29**
5	**97%**	***MSH2-GRHL2* rearrangement +LOH**	**No**	**Yes**	**Yes**	**34**
6	99%	No	No	50%	No	5
7	25%	-	-	0%	-	-
8	31%	No	No	70%	No	5
9	35%	No	*BRCA2* c.5946delT +likely LOH	10%	No	3
10	**-**	***MLH1* exon 19+ 3'UTR homozygous deletion**	**No**	**Yes**	**Yes**	**32**

In the no-cost extension year we plan to further evaluate IHC patterns in hypermutated tumors to determine if IHC may be a reliable screening assay to identify hypermutation.

Specific Aim 4: Implement diagnostic testing for hypermutation status in the UW-OncoPlex program for precision cancer medicine.

Aim 4, Subtask 1: *Establish a clinical trial that includes hypermutation testing by UW-OncoPlex with or without additional MSI-PCR/MSI-IHC tests depending on results of Aim 3*

Work on Aim 4 is currently is in progress and will be a focus in the no-cost extension year. In year 1 we obtained IRB human subjects approval for this work. We have begun to offer clinical UW-OncoPlex testing (**Table 3**) for prostate cancer patients with a total of 155 patients tested to date. Clinical reports are provided and results discussed directly with treating oncologists and urologists at a monthly precision tumor board led by Dr. Pritchard (**Figure 10**). We are evaluating treatment decision making in prostate cancer patients who have undergone UW-OncoPlex testing. We have published a manuscript describing our findings in the first 45 patients (see Appendix, Cheng et al. 2016 *Prostate*).

Table 3: UW-OncoPlex™ genes (assay version 5)

Tier 1: Currently actionable	ABL1	AKT1	ALK	AR	**ARAF**	ASXL1	ATM	AURKA	BCR	BRAF
	BRCA1	BRCA2	CALR	CCND1	CEBPA	**CSF3R**	**DDR2**	DNAJB1	DNMT3A	EGFR
	EML4	ERBB2	**ERCC2**	**ESR1**	FGFR2	FGFR3	FGFR4	FLT3	HIF1A	IDH1
	IDH2	JAK2	KIT	KRAS	MAP2K1	MET	MLL	MPL	**MTOR**	**MYD88**
	NOTCH1	NOTCH2	NPM1	NRAS	NTRK1	**NTRK2**	**NTRK3**	PALB2	PDGFRA	PIK3CA
	PML	PTEN	RARA	RET	ROS1	**SETBP1**	SMO	STK11	TP53	VHL
Tier 2: Actionable in the near future	ABL2	AKT2	AKT3	ARID1A	ATRX	AURKB	AXL	BAP1	BARD1	BCL2L11
	BCOR	**BCORL1**	BRIP1	CBL	CBLB	CCNE1	CDK4	CDK6	CDK8	CHEK1
	CHEK2	DAXX	ERBB3	ERBB4	FAM175A	**FANCA**	FBXW7	FGFR1	FLT1	FLT4
	GATA2	GATA3	GLI1	GNA11	GNAQ	GRM3	H3F3A	HDAC4	HRAS	IGF1R
	IKZF1	JAK3	KDM6A	KDR	KIF5B	MAP2K2	MAPK1	MC1R	MCL1	MDM2
	MDM4	MEN1	MITF	MLH1	MRE11A	MSH2	MSH6	MYC	MYCN	NBN
	NF1	NF2	NKX2-1	PAX5	PDGFRB	PHF6	PIK3R1	PMS2	POLD1	POLE
	RAD51C	RAD51D	RAF1	RB1	**RSPO2**	**RSPO3**	RUNX1	SHH	SMAD4	SMARCA4
	SRSF2	SUFU	SUZ12	TACSTD2	TET2	TMPRSS2	TSC1	TSC2	WT1	
Tier 3: Frequently mutated	APC	BAK1	BCL2	CBLC	**CBLC**	CDH1	**CDK12**	**CDK9**	CDKN1A	CDKN2A
	CHD1	CREBBP	CRLF2	CSF1R	CTNNB1	**CUX1**	DEPDC5	DOCK7	EPHA3	EPHA5
	EPHB2	EPHB6	ETV6	EZH2	**FKBP1A**	FOXA1	GAB2	GATA1	GNAS	GRIN2A
	HNF1A	IL7R	JAK1	MAP2K4	MED12	MIOS	MLH3	MTAP	MUTYH	MYCL1
	NPRL2	NPRL3	PAK1	PBRM1	PLK1	PLK3	PLK4	PRPF40B	PTCH1	PTPN11
	PTPRD	**RAC1**	**RAD21**	**RHEB**	RICTOR	RPS14	**RPTOR**	SF1	SF3B1	SMAD2
	SMAD3	SMARCB1	**SMC1A**	**SMC3**	SPOP	SPRY4	SRC	TACC3	**TET1**	**TET3**
	TFG	TGFBR2	TRRAP	U2AF1	U2AF65	ZBTB16	ZRSR2			
Germline pharmaco-genomics	ABCB1	ABCC4	ABCG2	CYP1B1	CYP2C19	CYP2C8	CYP2D6	CYP3A4	CYP3A5	DPYD
	EIF3A	ESR2	FCGR1A	FCGR2A	FCGR3A	GSTP1	ITPA	LRP2	MAN1B1	MTHFR
	NQO1	NRP2	SLC19A1	SLC22A2	SLCO1B3	SOD2	SULT1A1	TPMT	TYMS	TYR
	UGT1A1	UMPS								

*New genes on v5 in **BOLD**

http://tests.labmed.washington.edu/UW-OncoPlex
This is a clinically-available comprehensive gene sequencing platform co-developed and offered clinically by Dr. Pritchard's CLIA-certified genetics and solid tumors laboratory.

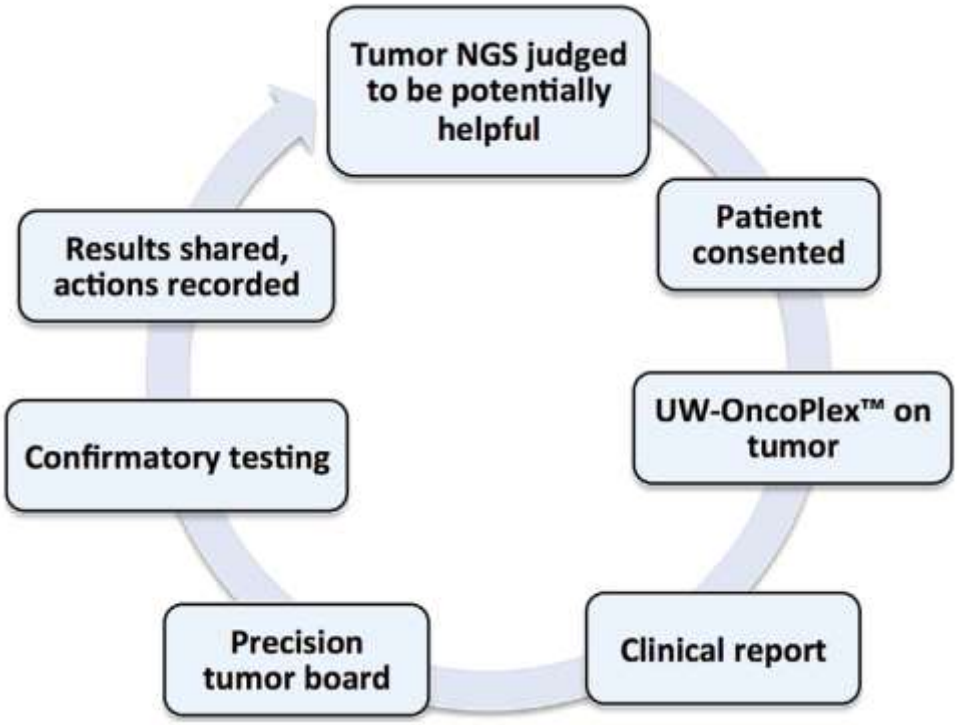


Figure 10: UW-OncoPlex Prostate Pilot Study Schema.

We plan to use this established assay and tumor board framework to formally test the role of hypermutation status as a precision biomarker. Recent work suggests that hypermutation and MSI due to DNA mismatch repair deficiency is a predictive biomarker for anti-programmed cell death 1 (PD-1) immunotherapy in several cancers (Le et al. N Engl J Med. 2015 372:2509-20). In collaboration with Dr. Michael Schweizer, we have established a protocol to screen men with prostate cancer for MSI using MSIplus as a qualifying test for enrollment on an anti-PD-1 therapeutic trial (durvalumab). Men who screen positive by MSIplus will have UW-OncoPlex testing done on tumor to determine hypermutation and MMR gene mutation status, as well as to confirm MSI. This trial is set to begin enrollment in late 2017.

Aim 4, Subtask 2: *Report hypermutation status results to medical oncologists in prostate cancer precision tumor board meetings and document treatment decisions and short-term outcomes.*

Work on this subtask was a focus in year 2 and will continue in year 3. We have already identified 10 of 155 patients (6.4%) with hypermutated prostate cancer at precision tumor board. 8/10 patients had clear MSI detected by our mSINGS method using UW-OncoPlex and all 10 patient had an underlying tumor *MMR* mutation with associated loss of heterozygosity or homozygous deletion, meaning bi-allelic inactivation (**Table 4**). Two patients had Lynch syndrome with a germline *MSH2* and *PMS2* mutation, respectively; the other 8 patients had double somatic MMR mutations. The discussion at precision tumor board suggested that the patient may be eligible for an anti-PD-1 checkpoint inhibitor immunotherapy trial. Recent work on MSI-high cancers indicates that hypermutation may predict response to anti-PD1 therapy.

One patient with an *MSH6* mutation and hypermutated MSI cancer received the PD-1 inhibitor pembrolizumab and achieved a partial PSA response before the drug needed to be discontinued due to side effects (**Figure 13**). In the no-cost extension year we anticipate identifying additional hypermutated patients through our study.

Table 4: Summary of hypermutated patients identified by UW-OncoPlex

MMR gene alteration	MSI Status by OncoPlex (using mSINGS)	Coding Mutations (per 1.2Mb)	Ductal Histology?
MSH6 frameshift + LOH	8/63 (13% unstable)	29	Yes
MSH2-GRHL2 rearrangement + LOH	21/65 (32% unstable)	34	Yes
MLH1 focal homozygous deletion	18/65 (28% unstable)	32	Yes
MSH2 p.Q61X germline + LOH	9/61 (15% unstable)	17	Unknown
MLH1 focal homozygous exon 19 to 3'UTR deletion	18/65 (27% unstable)	23	No
MSH2-AC022311.1 inversion + LOH	22/59 (37% unstable)	39	No
MSH2-CLK4 2;5 translocation + LOH	31/65 (48% unstable)	35	No
MSH2 inversion + LOH	26/65 (40% unstable)	29	No
MSH2 exon 1-6 + EPCAM del + LOH	20/64 (31% unstable)	22	No
PMS2 c.989-1G>T germline + LOH	6/64 (9% unstable)	24	No

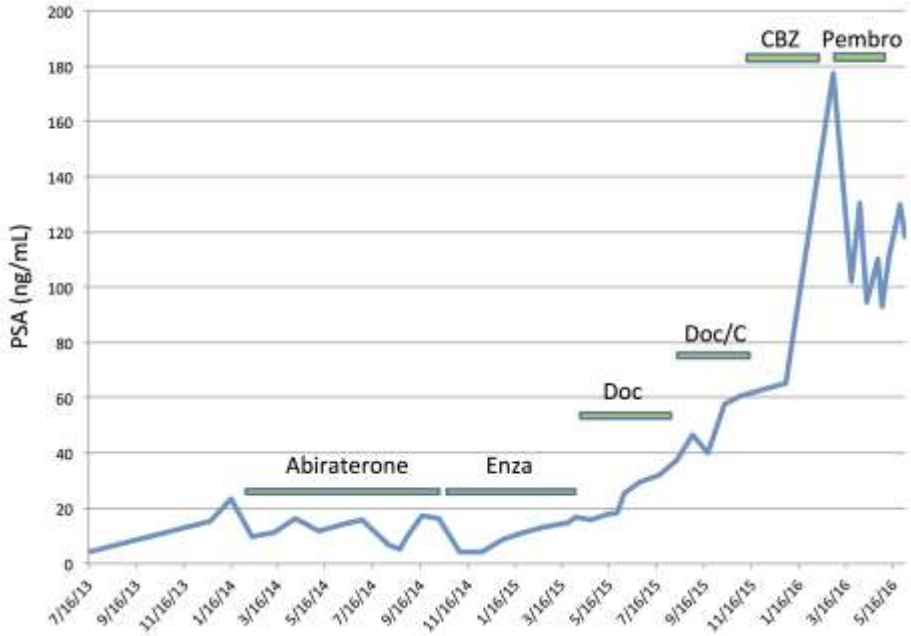


Figure 13: PSA response to checkpoint blockade immunotherapy in a patient with hypermutated prostate cancer. Prior to initiating pembrolizumab, patient had bone, adrenal and lymph node metastases, and a baseline PSA of 177.35 (ng/mL). A total of 3 cycles of pembrolizumab were administered before stopping due to an immune related adverse event requiring corticosteroids. Enza, enzalutamide; Doc, docetaxel; C, carboplatin; CBZ, cabazitaxel; Pembro, pembrolizumab.

3.3 What opportunities for training and professional development has the project provided?

Training-specific tasks from the approved SOW are given below. Detail related to training goals in the first year is provided in the section that follows.

Major Task: Training and educational development in prostate cancer research	**Months**	**Completed in Years 1-3?**
Subtask 1: Attend the Prostate Cancer Foundation Annual Retreat and the Association of Molecular Pathology Annual Conference	1-36	Yes, see *National Conferences and Committees section below*
Subtask 2: Present research at the monthly mentor group meetings, and at least once per year at Pacific Northwest Prostate Cancer SPORE research conferences	1-36	Yes, *Seminars* and *Interaction with Mentors sections below*
Subtask 3: Lead local prostate cancer precision tumor board, including review of genomic sequencing data and preparation of presentations that integrate prostate cancer patient clinical histories with genomic findings	1-36	Yes, see *Prostate Precision Tumor Board section* below
Subtask 4: Attend face-to-face meetings as part of the Stand-Up-To-Cancer (SU2C) prostate cancer dream team 2 to 3 times/year. Attend monthly conference calls for the SU2C prostate cancer dream team sequencing and analysis group	1-24	Yes, see *National Conferences and Committees section below*
Subtask 5: Lead local “pipeline” journal club focused on application of new genomic technologies in the clinic	1-36	Yes*, see Journal Club section below*
Subtask 6: Train senior pathology residents and fellows in the interpretation and clinical reporting of prostate-cancer focused precision diagnostics	1-36	Yes*, see Teaching section below*
Subtask 7: Serve as PI for the prostate cancer precision medicine component of the local “ACT-SMART” initiative as part of the institute for prostate cancer research	1-36	Yes
Subtask 8: Clinical reporting of UW-OncoPlex testing applied to advanced prostate cancer	12-36	Yes, *see prostate cancer precision tumor board and clinical duties below*
Subtask 9: Hands-on training in prostate cancer with Dr. True in pathology/immunohistochemistry and exposure to preclinical	1-36	Yes, see *Hand’s-on training*

prostate cancer models with Drs. Vessella and Morrissey.		section below
Milestone(s) Achieved: Publication of original research	24-36	Yes, *see appendices for publications in the first year*
Milestone(s) Achieved: Presentation of project data at a national meeting	12-36	Yes, see *National Conferences and Committees section below*

National Conferences and Committees: In years 1-3 I attended the Prostate Cancer Foundation annual retreat where I presented work on mechanisms of hypermutation in advanced prostate cancer both years. I also attended the Prostate Cancer Foundation Coffey-Holden Prostate Cancer Academy Meeting in Years 1, 2, and 3. This 3-day invite-only meeting was a think tank of leading prostate cancer researchers focused on the question of oligo-metastatic disease. I was part of the program organizing committee for the 2016 Coffey-Holden Prostate Cancer Academy meeting in year 2, where I organized a session on DNA repair deficiency in advanced prostate cancer. This session in included talks from international experts that invited for my moderated session, including Dr. Rob Bristow and Dr. Joaquin Mateo, and Dr. Heather Cheng. In year 3 I was invited to speak on my work on the hypermutated subtype of prostate cancer at the Prostate Cancer Foundation annual retreat in the main session. I have attended the Association of Molecular Pathology Annual Conference, which is my primary professional society. I was invited to give an opening plenary session at the 2015 Association of Molecular Pathology Annual Conference on the topic of bioinformatics as a new area for the clinical laboratory. I have also been invited to give the Plenary Session at the upcoming 2017 Association of Molecular Pathology Annual Conference on the topic of the intersection of germline and somatic genetic testing and new areas of clinical utility. I year 3 I was also invited to share the keynote address with Dr. Mike Caliguiri, the incoming president of AACR, at the 2017 Association of Clinical Pathology annual meeting. I also attended Stand Up to Cancer (SU2C) Prostate Cancer International Dream Team face-to-face meetings and participated in monthly SU2C sequencing and analysis conference calls. In Year 2 I was invited to present in Chicago as part of the SU2C/PCF international dream team site visit on my work in DNA repair mutations in advanced prostate cancer. As part of my involvement with the SU2C Prostate Cancer International Dream Team I analyzed genomic data, prepared figures for publication, and gave a formal presentation to the SU2C team members. In Year 2 I also attended the DOD IMPACT meeting in Baltimore where a presented a poster on the work achieved to date through this award. My poster was one of only 4 selected for a video interview that was posted on the CDMRP website and on YouTube to highlight the work of the CDMRP PCRP program . In year 3 I was invited to be on the Advanced Prostate Cancer Consensus Conference (APCCC) committee. This is a prestigious international committee that meets in Switzerland every two years to vote on practice recommendations for advanced prostate cancer. I was one of only 2 pathologists to be invited to participate in the consensus conference. I was asked to draft questions regarding prostate cancer genetics in clinical

practice, including questions on clinical testing for hypermutation and mismatch repair deficiency.

Seminars: I continue to attend and present at the weekly Pacific Northwest Prostate Cancer SPORE conference series. For example, in Year 1 I presented a SPORE talk my work on Aim 1 of this research was presented in addition to my collaborative with the SU2C international dream team. In September of Year 2 I presented at SPORE again with an updated on the work completed to date as part of this project. I also attend weekly Laboratory Medicine grand rounds and Laboratory Medicine research rounds which include a wide range of topics related to clinical diagnostic medicine. Finally, I attend weekly genetics seminars hosted by our division.

Teaching: In year 1 and 2 of funding I have trained a total of 6 senior clinical pathology residents, 3 molecular genetic pathology fellows, and 2 junior molecular pathology faculty (Dr. Eric Konnick and Dr. Tina Lockwood) in the clinical interpretation and reporting of genomic testing for prostate cancer. I have continued to be an active mentor to our molecular genetic pathology (MGP) fellowship director, MGP fellows, and chief resident in prostate cancer-related molecular diagnostics. In addition, I have mentored 4 medical genetics senior residents in molecular oncology diagnostics, including one resident (Dr. Mari Tokita) who spent 9 months of dedicated time in my research laboratory developing circulating tumor DNA diagnostics methods. As part of this work she helped to assemble ctDNA samples for clinical assay validation from patients with metastatic prostate cancer. As co-director of the genetics and solid tumors laboratory at the UW I have also mentored 16 junior clinical pathology residents in 4-week basic genetics training rotations.

Journal Club: I continue to lead the “pipeline” monthly journal club at UW which is attended by about 20-30 faculty and senior trainees and is focused on genomic technologies applied in the clinic. I have recruited several speakers in year 1 of funding. Examples include a speaker (Mary Goldman) who works for the UCSC genome browser and outlined new tools for cancer data visualization, and a speaker who discussed new methods for gene fusion analysis in cancer, including *TMPRSS2-ERG* fusion detection in prostate cancer (David Wu). This is a forum where the Molecular Genetic Pathology fellows I mentor are required to present at least once per year.

Prostate Precision Tumor Board**:** For the entirety of this award period I have lead the local prostate precision tumor board at the University of Washington where we review genome sequencing data from advanced prostate cancer patients and make treatment recommendations to treating oncologists. This activity is a substantial effort in which I prepare detailed PowerPoint presentations for each patient and do a thorough literature review to identify potential or actual therapeutic implications for genomic findings. This monthly tumor board is attended by GU medical oncologists, pathologists (including co-mentor Dr. Larry True), urologists, research coordinators, genetic counselors, and trainees including our junior faculty and molecular genetic pathology fellows. This forum continues to grow in popularity as molecular findings in prostate cancer begin to be using in practice and it is very well-attended

Clinical Duties**:** I am a primary faculty member responsible for clinical reporting of BROCA and UW-OncoPlex tests that are available through my CLIA-certified laboratory. I have already trained 2 other junior molecular pathology faculty to assist me, and will continue to mentor and

train new junior faculty to assist with these clinical duties. My clinical work includes close mentorship from Dr. Mary-Claire King, who personally consults on all BROCA cases. We have weekly "signout" meetings that last 1 to 2 hours. I interpret and write clinical reports for approximately 1,000 genomic sequencing cases per year. As part of my clinical work I interpret and report on UW-OncoPlex clinical testing for all prostate cancer patients tested.

***Grant Writing*:** In collaboration with Dr. Peter Nelson and Dr. Bruce Montgomery, I am a partnering PI for a DOD IMPACT proposal focused on minimally invasive methods to detect DNA repair defects in advanced prostate cancer. I have also submitted a DOD IMPACT proposal as initiating PI in collaboration with partnering PIs Dr. Joaquin Mateo and Dr. David Olmos focusing on qualifying DNA repair defects as biomarkers for standard and emerging therapies. I have a submitted an R01 application in collaboration with Heather Hampel at The Ohio State University focused on genomic sequencing as a tool for Lynch syndrome screening. This application benefits from the work we have done to develop robust methods to detect hypermutation and MSI in cancer. I am a co-investigator on a pending NIH R21 award (PI Steve Salipante) focused on novel methods of detecting MSI. Finally, I am key collaborator on a prostate cancer foundation challenge award application focused on MMR deficiency and immunotherapy.

***Hand's-on training*:** With Dr. True I have received personal tutorials in prostate cancer pathology, including one-on-one formal lectures followed by teaching slide review. In addition, Dr. True provides helpful feedback on prostate pathology-related issues at the monthly prostate precision tumor board that I lead. I also receive 'hand's on' exposure to preclinical prostate cancer models with Drs. Eva Corey, Robert Vessella and Colm Morrissey in the University of Washington GU Cancer Research Laboratory.

Interaction with Mentors: I am **co-mentored by Dr. Larry True and Dr. Mary-Claire King** for this training award. Dr. True is an internationally prominent genitourinary pathologist with over 30 years-experience mentoring junior academic researchers. He and I have worked together since 2000, and he has effectively mentored me in aspects of prostate cancer pathology, and molecular biomarker development. For example, I served on the NCI Tissue-Based biomarker subcommittee of the Investigational Drug Steering Committee that Dr. True chaired. Dr. King is an internationally famous geneticist who discovered the *BRCA1* locus, and has mentored dozens of highly successful faculty in the area of cancer genetics. She has mentored me since 1998 as part of the medical scientist training program and has been an even closer ongoing mentor to me over the past 4 years in the clinical implementation of the BROCA assay that was developed in her laboratory by Dr. Tom Walsh. I meet more than once a month at length with Dr. King to discuss BROCA test results and discuss research directions, and I meet with Dr. True at the monthly prostate cancer precision tumor board. In addition, I meet with Dr. True for formal prostate cancer pathology training.

3.4 How were the results disseminated to communities of interest?

Through the Institute for Prostate Cancer Research (IPCR) we have reached out to patient advocates in the region. This has included a formal presentation on prostate cancer precision medicine that I gave to a lay audience for the IPCR on the topic of the UW-OncoPlex program for prostate cancer precision medicine. In year 2 we aired a segment on TV that highlights the prostate cancer precision tumor board that I lead. Also, in year 2 at the Innovative Minds in

Prostate Cancer Today (IMPACT) meeting I was filmed at my poster for segment to be posted for patients and researcher on the CDMRP website. In year 3, work from this award was selected for highlight on the CDMRP website with a detailed write-up.

3.5 What do you plan to do during the next reporting period to accomplish the goals?

Plans in the no-cost extension year

Research-specific tasks

- Evaluate performance characteristics of MSIplus for prostate cancer and determine the optimal loci.
- Continue prostate cancer pilot project using clinical UW-OncoPlex for men with prostate cancer. Use experience gained from clinical testing to inform a future clinical trial for men with hypermutated prostate cancer.
- Incorporate circulating tumor DNA testing (ctDNA) into the UW-OncoPlex pilot study and evaluate the performance of ctDNA compared to matched tumor tissue testing as the second phase of the pilot study.
- Continue to evaluate treatment decision making in prostate cancer patients who have undergone UW-OncoPlex testing through review with treating oncologists at the precision tumor board. The primary measured outcome will be treatment decisions specifically influenced by hypermutation status results. We will use this data to plan future clinical trials to more formally test the role of hypermutation status as a precision biomarker.
- Continue to evaluate histologic correlates that may be predictive of hypermutation status and MMR deficiency including ductal histology.
- Begin enrolling patients on clinical trial of PD-L1 inhibitor durvalumab using MSIplus and UW-OncoPlex to pre-screen patients that have hypermutated prostate tumors harboring MSI.

Training-specific tasks

- Attend PCF annual meeting and AMP annual meetings as well as SU2C prostate international dream team face-to-face meetings.
- Continue to present data at SPORE conference and at national conferences.
- Continue resident and fellow training of genomic testing in prostate cancer
- Continue hand's on training meetings with Dr. True in prostate cancer pathology
- Continue frequent genomic sequencing signout sessions in cancer genetics with Dr. Mary-Claire King (about 2 to 3 times per month).
- Continue to lead monthly Prostate Precision Tumor Board
- Continue to lead monthly pipeline journal club
- Continue grant writing activities
- Continue activity on national and international prostate cancer committees

4. IMPACT

4.1 What was the impact on the development of the principal discipline(s) of the project?

This research has led to the discovery of the mechanism of hypermutation in advanced prostate cancer. We found that complex somatic *MSH2* and *MSH6* mismatch DNA repair mutations resulting in microsatellite instability are the chief cause of hypermutation. We also found that hypermutation is more common in advanced prostate cancer than previously expected, with 10/103 (10%) patients identified in our series. Our discovery identifies parallels and differences in the mechanisms of hypermutation in prostate cancer compared with other microsatellite instability-associated cancers. Our findings have important implications for prognosis and treatment. If hypermutation can be targeted, a substantial minority of patients with advanced prostate cancer may benefit. For example, cancers with mismatch DNA repair deficiency have recently been shown to be responsive to anti-PD-1 immunotherapy. Our research has also facilitated microsatellite instability and immunohistochemistry-based testing as screening tools for hypermutation in advanced prostate cancer, as well as identified an important histologic correlate that may facilitate identification of hypermutated cancers.

Our work has also led to the development of highly innovative and robust methods to detect microsatellite instability (MSI) that is associated with hypermutation. We recently developed the "mSINGS" method for detection of MSI directly from NGS. This has facilitated MSI analysis of exome data from the SU2C prostate cancer international dream team consortium, proving that all hypermutated prostate cancer cases in that series also have MSI associated with underlying mismatch DNA repair mutations. Importantly, our work as part of this award has led to the approval of a clinical trial of the PD-L1 inhibitor durvalumab in men with hypermutated MSI prostate cancer. This trial will use MSIplus and UW-OncoPlex to identify eligible men with hypermutated prostate cancers.

4.2 What was the impact on other disciplines?

Our work builds bridges between research in colorectal and endometrial cancer and research in prostate cancer. Hypermutation and MSI are well-studied in colorectal and endometrial cancer. We have applied the mSINGS method we developed to both colorectal and endometrial cancer, resulting in the first ever tumor-based DNA sequencing test for Lynch syndrome, ColoSeq Tumor. We collaborate closely with colleagues at the Ohio State University on Lynch syndrome screening research (Heather Hampel and Albert de la Chapelle), resulting in a recent NIH R01 grant submission that harnesses the mSINGS method. In year 3 we performed tumor sequencing via UW-OncoPlex of nearly 500 colorectal cancer cases prospectively collected in Ohio state to evaluate if tumor sequencing can be effectively used a screening test for MMR mutation, MSI status, hypermutation, and other predictive biomarkers. This work has led to a manuscript currently in review.

Our work also builds bridges with basic science disciplines. The mSINGS method we developed was recently used to profile the landscape of microsatellite instability across 18 different cancer types, providing important insights into the biology of MSI.

4.3 What was the impact on technology transfer?

Nothing to Report

4.4 What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS

5.1 Changes in approach and reasons for change

Due to cost constraints we were required to slightly modify our planned experimental design for aim 2 to evaluate only 3 different therapies in the hypermutated and non-hypermutated LuCaP xenograft models. The three therapies chosen are docetaxel, carboplatin, and 5-FU. Note that this change does not affect the budget of this award because no vertebrate animal work is funded through this training award.

5.2 Actual or anticipated problems or delays and actions or plans to resolve them

Based on prior experience by the GU cancer research laboratory we anticipated potential problems with carboplatin toxicity in future LuCaP xenograft animal studies. This is particularly a problem for the LuCaP 58 hypermutated line in which frequent ulceration may occur. To address this, we lowered the dose and dosing frequency of carboplatin for this arm of the study.

5.3 Changes that had a significant impact on expenditures

Nothing to report.

5.4 Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. PRODUCTS

6.1 Publications, conference papers, and presentations

6.11 Journal publications

Pritchard CC (corresponding author), Morrissey C, Kumar A, Zhang X, Smith C, Coleman I, Salipante SJ, Milbank J, Tait JF, Corey E, Vessella RL, Walsh T, Shendure J, Nelson PS; Complex *MSH2* and *MSH6* Mutations in Hypermutated Microsatellite Unstable Advanced Prostate Cancer; *Nature Communications*; 5: 2014; 4988; published; acknowledgement of federal support (yes).
See Appendix 1 and 2

Robinson D, Van Allen EM, Wu Y, Schultz N., Lonigro RJ, Mosquera J, Montgomery R, Taplin ME, **Pritchard CC (co-second author)**, Attard G, Beltran H, Abida WM, Bradley RK, Vinson J, Cao X, Vats P, Kunju LP, Hussain M, Feng FY, Tomlins SA, Cooney KA, Smith DC, Brennan C, Siddiqui J, Mehra R, Scher HI, Chen Y, Rathkopf DE, Morris MJ, Solomon SB, Durack JC,

Reuter VE, Gopalan A, Gao J, Loda M, Lis RT, Bowden M, Balk SP, Gaviola G, Sougnez C, Gupta M, Yu EY, Mostaghel EA, Cheng HH, Chew FS, True LD, Plymate SR, Dvinge H, Ferraldeschi R, Flohr P, Miranda S, Zafeiriou Z, Tunariu N, Mateo J, Demichelis F, Elemento O, Robinson BD, Sboner A, Schiffman MA, Nanus DM, Tagawa ST, Sigaras A, Eng KW, Heath E, Pienta KJ, Kantoff P, de Bono JS, Rubin MA, Nelson PS, Garraway LA, Sawyers CL, Chinnaiyan AM; Integrative clinical sequencing analysis of metastatic castration resistant prostate cancer reveals a high frequency of clinical actionability; *Cell*. 161: 2015; 1215–1228; published; acknowledgement of federal support (yes).
See Appendix 3

Hempelmann JA, Scroggins SM, **Pritchard CC**, Salipante SJ; MSIplus: integrated colorectal cancer molecular testing by next-generation sequencing; *Journal of Molecular Diagnostics*. 17: 2015; 705-14; published; acknowledgement of federal support (yes).
See Appendix 4

Cheng HH, **Pritchard CC**, Boyd T, Nelson PS, Montgomery B. Biallelic Inactivation of BRCA2 in Platinum-sensitive Metastatic Castration-resistant Prostate Cancer. *European Journal of Urology*. 69: 2016; 992-5. published; acknowledgement of federal support (yes).
See Appendix 5

Van Allen EM, Robinson D, Morrissey C, **Pritchard C**, Carter SL, Rosenberg M, McKenna A, Chinnaiyan A, Garraway L, Nelson PS. A Comparative Assessment of Clinical Whole Exome and Transcrip-tome Profiling Across Sequencing Centers: Implications for Precision Cancer Medicine. *Oncotarget*. 2016: Epub ahead of print. PMID:27167109. published; acknowledgement of federal support (yes).

Cowen (Shiovitz) S, Turner EH, Beightol MB, Jacobson A, Gooley TA, Salipante SJ, Haraldsdottir S, Smith C, Scroggins S, Tait JF, Grady WHM, Lin EH, Cohn, DE, Goodfellow PJ, Arnold MW, Chapelle Adl, Pearlman R, Hampel H, **and Pritchard CC (senior author)**. Frequent PIK3CA Mutations in Colorectal and Endometrial Tumors with 2 or More Somatic Mutations in Mismatch Repair Genes. *Gastroenterology* 151: 2016; 440-447. published; acknowledgement of federal support (yes).

Cheng HH, Klemfuss N, Montgomery B, Higano CS, Schweizer MT, Mostaghel EA, McFerrin LG, Yu EY, Nelson PS, **and Pritchard CC (senior author)**. Pilot study of clinical targeted next generation sequencing for prostate cancer: consequences for treatment and genetic counseling. *Prostate*. 76: 2016; 1303-11. published; acknowledgement of federal support (yes).
See Appendix 6

Mateo J, Boysen G, Barbieri CE, Bryant HE, Castro E, Nelson PS, Olmos D, **Pritchard CC**, Rubin MA, de Bono JS. DNA Repair in Prostate Cancer: Biology and Clinical Implications. *European Journal of Urology*. Epub ahead of print: 2016; PMID:27590317. published; acknowledgement of federal support (yes).
See Appendix 7

Pritchard CC (first author), Mateo J, Walsh MF, De Sarkar N, Abida W, Beltran H, Garofalo A, Gulati R, Carreira S, Eeles R, Elemento O, Rubin MA, Robinson D, Lonigro R, Hussein M, Chinnaiyan A, Vinson J, Filipenko J, Garraway L, Taplin M-E, AlDubayan S, Han GC, Beightol

M, Morrissey C, Noteboom J, Nghiem B, Cheng HH, Montgomery B, Walsh T, Casadei S, Vijai J, Scher HI, Sawyers C, Schultz N, Kantoff P, Solit D, Robson M, Van Allen EM, Offit K, DeBono J, and Nelson PS. Inherited DNA-Repair Gene Mutations in Men with Metastatic Prostate Cancer. *New England Journal of Medicine*. 375: 2016; 443-53. published; acknowledgement of federal support (yes).
See Appendix 8

Hause RJ, **Pritchard CC**, Shendure J, Salipante SJ. Classification and characterization of microsatellite instability across 18 cancer types. *Nature Medicine*. 2016: Epub ahead of print; PMID:27694933. published; acknowledgement of federal support (yes).
See Appendix 9

Schweizer MT, Cheng HH, Tretiakova MS, Vakar-Lopez F, Klemfuss N, Konnick EQ, Mostaghel EA, Nelson PS, Yu EY, Montgomery RC, True LD, **and Pritchard CC**. Mismatch Repair Deficiency May Be Common in Ductal Adenocarcinoma of the Prostate. (2016). Oncotarget. 7:82504-82510. PMID:27756888. published; acknowledgement of federal support (yes).
See Appendix 10

Cheng HH, **Pritchard CC**, Montgomery B, Lin DW, Nelson PS. Prostate Cancer Screening in a New Era of Genetics. Clin Genitourin Cancer. (2017) Epub ahead of print. PMID: 28697982. published; acknowledgement of federal support (yes).
See Appendix 11

Gillessen S, Attard G, Beer TM, Beltran H, Bossi A, Bristow R, Carver B, Castellano D, Chung BH, Clarke N, Daugaard G, Davis ID, de Bono J, Dos Reis RB, Drake CG, Eeles R, Efstathiou E, Evans CP, Fanti S, Feng F, Fizazi K, Frydenberg M, Gleave M, Halabi S, Heidenreich A, Higano CS, James N, Kantoff P, Kellokumpu-Lehtinen PL, Khauli RB, Kramer G, Logothetis C, Maluf F, Morgans AK, Morris MJ, Mottet N, Murthy V, Oh W, Ost P, Padhani AR, Parker C, **Pritchard CC**, Roach M, Rubin MA, Ryan C, Saad F, Sartor O, Scher H, Sella A, Shore N, Smith M, Soule H, Sternberg CN, Suzuki H, Sweeney C, Sydes MR, Tannock I, Tombal B, Valdagni R, Wiegel T, Omlin A. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol. (2017) Epub ahead of print. PMID: 28655541. published; acknowledgement of federal support (no).
See Appendix 12

Guedes LB, Antonarakis ES, Schweizer MT, Mirkheshti N, Almutairi F, Park JC, Glavaris S, Hicks J, Eisenberger MA, De Marzo AM, Isaacs WB, Eshleman JR, **Pritchard CC*,** Lotan TL* MSH2 Loss in Primary Prostate Cancer. (2017). Clin. Cancer Res. Epub ahead of print. PMID:28790115 *Co-senior author. published; acknowledgement of federal support (yes).
See Appendix 13

6.12 Books or other non-periodical, one-time publications.

Friedlander TW, Pritchard CC, Beltran H. Personalizing Therapy for Metastatic Prostate Cancer: The Role of Solid and Liquid Tumor Biopsies. Am Soc Clin Oncol Educ Book. (2017). 37:358-369. PMID: 28561699. published; acknowledgement of federal support (no).

6.13 Other publications, conference papers, and presentations.

Pritchard CC, Morrissey C, Kumar A, Zhang X, Smith C, Coleman I, Salipante S, Grady WM, Tait JF, Vessella R, Walsh T, Shendure J, and Nelson PS. Mechanisms of Microsatellite Instability in Hypermutated Advanced Prostate Cancer. (2014) Prostate Cancer Foundation Annual Scientific Retreat.

Salipante S, Scroggins S, Hampel HL, Turner EH, and **Pritchard CC**. Microsatellite Instability Detection By Next-Generation Sequencing. (2014) Academy of Clinical Laboratory and Physician Scientists Annual Meeting.

Cheng HC, Klemfuss N, Montgomery B, Higano CS, Schweizer MT, Mostaghel E, Yu EY, Nelson PS, **and Pritchard CC**. Pilot study of clinical targeted next generation sequencing for prostate cancer: treatment and genetic counseling actionability. (2015) Prostate Cancer Foundation Annual Scientific Retreat.

Shiovitz S, Turner EH, Beightol MB, Jacobson A, Gooley TA, Salipante SJ, Haraldsdottir S, Smith C, Scroggins S, Tait JF, Grady WHM, Lin EH, Cohn, DE, Goodfellow PJ, Arnold MW, Chapelle Adl, Pearlman R, Hampel H, **and Pritchard CC**. *PIK3CA* mutations in colorectal and endometrial cancer with double somatic mismatch repair mutations compared to Lynch syndrome. (2015) American Society of Clinical Oncology Annual Meeting.

Cheng HH, **Pritchard CC**, Boyd T, Nelson PS, and Montgomery B. Biallelic Inactivation of *BRCA2* in Platinum-sensitive, Metastatic Castration Resistant Prostate Cancer. (2016). American Society of Clinical Oncology Genitourinary Cancers Symposium Meeting.

Nelson PS, Mateo J, Beltran H, De Sarkar N, Elemento O, Rubin MA, Vinson J, Filipenko J, Robinson DR, Chinnaiyan A, Garraway L, Van Allen EM, Garofalo A, Taplin M-E, Garraway LA, Carreira S, Montgomery RB, Morrissey C, Cheng HH, DeBono JS, **and Pritchard CC**. (2016). American Society of Clinical Oncology (ASCO) Annual Meeting. [Selected for oral platform presentation with discussant as ASCO]

Pritchard CC, Morrissey C, Cheng HH, Salipante S, True L, Schweizer M, Klemfuss N, Montgomery RB, Corey E, Nelson PS. Hypermutation in Advanced Prostate Cancer: From Mechanism to Implementation of Genomic Testing for Precision Therapy. (2016) DOD Prostate Cancer Young Investigators IMPACT meeting, Baltimore MD.

Shirts BH, Konnick EQ, Jacobson A, Garrett L, Hampel H, Pearlman R, King MC, Walsh T, **and Pritchard CC**. Using somatic mutations to classify pathogenic Lynch syndrome variants (2016) Association for Molecular Pathology annual meeting.

De Sarkar N, **Pritchard CC**, Nelson P. "Inherited Deleterious Germline Variants in Men with Prostate Cancer Identified by Whole Exome Sequencing" (2016) American Society for Human Genetics (ASHG) annual meeting. [Selected for oral platform presentation at ASHG]

Cheng HH, Montgomery B, Schweizer M, Mosteghel E, Yu EY, Mandell J, Walsh T, **Pritchard CC**, Nelson PS, King M-C. Through the Prostate Cancer Looking Glass: characterizing cancer-associated germline mutations through a prostate cancer family study, registry and prostate cancer genetics clinic. (2016) Prostate Cancer Foundation Annual Scientific Retreat.

Schweizer MT, Cheng HH, Tretiakova MS, Vakar-Lopez F, Klemfuss N, Konnick EQ, Mostaghel EA, Nelson PS, Yu EY, Montgomery RC, True LD, **and Pritchard CC**. Mismatch Repair Deficiency is Common in Ductal Adenocarcinoma of the Prostate. (2016) Prostate Cancer Foundation Annual Scientific Retreat.

6.2 Website(s) or other Internet site(s)

Nothing to report.

6.3 Technologies or techniques

We have developed the mSINGS method for detection of microsatellite instability from targeted next-generation sequencing data. The source code for this bioinformatics method is freely available for academic users and can be found at: https://bitbucket.org/uwlabmed/msings. This source code has already been shared with multiple national and international researchers.

6.4 Inventions, patent applications, and/or licenses

Nothing to report.

6.5 Other Products

The genomic sequencing dataset we generated as part of the research on aim 1 to identify mechanisms of hypermutation is publically available at GenBank/EMBL/DDBJ under the accession code SRP044943.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7.1 What individuals have worked on the project?

Name: Colin C. Pritchard
Project Role: PI
Researcher Identifier: ORCID ID: 0000-0002-7424-2956)
Nearest person month worked: 5
Contribution to Project: Dr. Pritchard has obtained funding support, designed experiments, and written manuscripts related to this work
Funding Support: (this award)

Name: Robert Livingston
Project Role: Senior Research Scientist
Nearest person month worked: 1

Contribution to Project: Dr. Livingston has coordinated coordinating genomic sequencing library preparation and assisted in data analysis.
Funding Support: (this award)

Name: Mallory Beightol
Project Role: Research Technician
Nearest person month worked: 3
Contribution to Project: Ms. Beightol has helped perform genomic sequencing assays including BROCA and UW-OncoPlex.
Funding Support: Institutional, PNW Prostate Cancer SPORE pilot funds

7.2 Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Colin Pritchard: Changes in current support since last reporting period:

Title: Characterizing the Hypermutated Subtype of Advanced Prostate Cancer as a Predictive Biomarker for Precision Medicine

Time Commitment: 1.80 calendar months

Supporting Agency: Department of Defense

Contracting/Grants Officer: Jennifer Shankle

Address of Funding Agency: USAMRAA, 820 Chandler St, Ft. Detrick, MD 21702-5014

Performance Period: 09/15/14 – 09/14/18

Level of Funding: $567,859

Project Goal: The goal of this research is to characterize the mechanisms leading to hypermutated prostate cancer and to integrate tumor hypermutation status with clinical decision making and therapy to improve the care of men with advanced prostate cancer. Using next-generation sequencing approaches my colleagues at the University of Washington recently discovered that 10-20% of advanced prostate cancers are hypermutated. Hypermutation was subsequently observed in primary prostate cancer. Prostate cancer hypermutation is a promising target for precision therapy, but the mechanisms leading to hypermutation, optimal methods to measure hypermutation status in the clinic, and clinical implications for prostate cancer patients are not yet understood.

Specific Aims: Aim 4: Implement diagnostic testing for hypermutation status in the UW-OncoPlex program for precision cancer medicine" is ongoing. Pembrolizumab has recently received approval for hypermutated tumors with MSI, representing the first molecular-biomarker driven therapy in prostate cancer.

Overlap: none

Title: Advanced development and validation of targeted molecular counting methods for precise and ultrasensitive quantitation of low prevalence somatic mutations

Time Commitment: 0.60 calendar months

Supporting Agency: National Institute of Health

Contracting/Grants Officer: Angela Walters

Address of Funding Agency: 9000 Rockville Pike, Bethesda, Maryland 20892

Performance Period: 05/01/2015 – 04/30/2018

Level of Funding: $295,570

Project Goal: The ultrasensitive detection of clinically relevant somatic alterations in cancer genomes has great potential for impacting patient care, e.g. for early detection, establishing diagnoses, refining prognoses, guiding treatment, and monitoring recurrence. However, current technologies are poorly suited to the robust detection of somatic mutations present at very low frequencies (<1%). Massively parallel sequencing represents an advantageous path forward, but its sensitivity to detect very rare events is fundamentally constrained by the sequencing error rate. We have recently developed a new experimental paradigm that overcomes this limitation.

Specific Aims: In our approach, each copy of a target sequence that is present in a sample is molecularly tagged during the first cycle of a multiplex capture reaction with a unique random sequence. After amplification, target amplicons and their corresponding molecular tags are subjected to massively parallel sequencing. During analysis, the molecular tags are used to associate sequence reads sharing a common origin. Through oversampling, reads bearing the same molecular tag error-correct one another to yield an independent haploid consensus for each progenitor molecule.

Overlap: none

7.3 What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

Appendix 1: Reprint of Pritchard CC et al.; Complex *MSH2* and *MSH6* Mutations in Hypermutated Microsatellite Unstable Advanced Prostate Cancer; *Nature Communications*; 5: 2014; 4988.

Appendix 2: Supplemental Figures from Pritchard CC et al.; Complex *MSH2* and *MSH6* Mutations in Hypermutated Microsatellite Unstable Advanced Prostate Cancer; *Nature Communications*; 5: 2014; 4988.

Appendix 3: Reprint of Robinson D et al.; Integrative clinical sequencing analysis of metastatic castration resistant prostate cancer reveals a high frequency of clinical actionability; *Cell*. 161: 2015; 1215–1228.

Appendix 4: Reprint of Hempelmann JA et al.; MSIplus: integrated colorectal cancer molecular testing by next-generation sequencing; *Journal of Molecular Diagnostics*. 17: 2015; 705-14.

Appendix 5: Cheng HH et al. Biallelic Inactivation of BRCA2 in Platinum-sensitive Metastatic Castration-resistant Prostate Cancer. *European Journal of Urology*. 69: 2016; 992-5.

Appendix 6: Reprint of Cheng HH et al. Pilot study of clinical targeted next generation sequencing for prostate cancer: consequences for treatment and genetic counseling. *Prostate*. 76: 2016; 1303-11.

Appendix 7: Reprint of Mateo J et al. DNA Repair in Prostate Cancer: Biology and Clinical Implications. *European Journal of Urology*. Epub ahead of print: 2016; PMID:27590317.

Appendix 8: Reprint of Pritchard CC et al. Inherited DNA-Repair Gene Mutations in Men with Metastatic Prostate Cancer. *New England Journal of Medicine*. 375: 2016; 443-53.

Appendix 9: Reprint of Hause RJ et al. Classification and characterization of microsatellite instability across 18 cancer types. *Nature Medicine*. 2016: Epub ahead of print; PMID:27694933.

Appendix 10: Reprint of Schweizer MT et al.. Mismatch Repair Deficiency May Be Common in Ductal Adenocarcinoma of the Prostate. (2016). Oncotarget. 7:82504-82510. PMID:27756888.

Appendix 11: Reprint of Cheng HH et al. Clin Genitourin Cancer. (2017) Epub ahead of print. PMID: 28697982.

Appendix 12: Reprint of Gillessen S et al. The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol. (2017) Epub ahead of print. PMID: 28655541.

Appendix 13: Reprint of Guedes LB et al. MSH2 Loss in Primary Prostate Cancer. (2017). Clin. Cancer Res. Epub ahead of print. PMID:28790115 *Co-senior author.

nature COMMUNICATIONS

ARTICLE

Received 13 Mar 2014 | Accepted 14 Aug 2014 | Published 25 Sep 2014

DOI: 10.1038/ncomms5988 OPEN

Complex *MSH2* and *MSH6* mutations in hypermutated microsatellite unstable advanced prostate cancer

Colin C. Pritchard[1], Colm Morrissey[2], Akash Kumar[3], Xiaotun Zhang[2], Christina Smith[1], Ilsa Coleman[4], Stephen J. Salipante[1,3], Jennifer Milbank[3], Ming Yu[5], William M. Grady[5], Jonathan F. Tait[1], Eva Corey[2], Robert L. Vessella[2], Tom Walsh[6], Jay Shendure[3] & Peter S. Nelson[4]

A hypermutated subtype of advanced prostate cancer was recently described, but prevalence and mechanisms have not been well-characterized. Here we find that 12% (7 of 60) of advanced prostate cancers are hypermutated, and that all hypermutated cancers have mismatch repair gene mutations and microsatellite instability (MSI). Mutations are frequently complex *MSH2* or *MSH6* structural rearrangements rather than *MLH1* epigenetic silencing. Our findings identify parallels and differences in the mechanisms of hypermutation in prostate cancer compared with other MSI-associated cancers.

[1] Department of Laboratory Medicine, University of Washington, Seattle, Washington 98195, USA. [2] Department of Urology, University of Washington, Seattle, Washington 98195, USA. [3] Department of Genome Sciences, University of Washington, Seattle, Washington 98195, USA. [4] Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109, USA. [5] Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109, USA. [6] Division of Medical Genetics, Department of Medicine, University of Washington, Seattle, Washington 98195, USA. Correspondence and requests for materials should be addressed to C.C.P. (email: cpritch@uw.edu).

© 2014 Macmillan Publishers Limited. All rights reserved.

Recently exome sequencing of metastatic prostate cancers revealed that a subset of patients harboured tumors with markedly elevated single-nucleotide mutation rates, defining a new hypermutated subtype[1]. This phenotype was subsequently observed in primary prostate cancer in a tumour that harboured an *MSH6* mutation[2]. However, mechanisms that lead to hypermutation and the prevalence of this distinct subtype have not been completely defined. Comprehensive cancer genomics efforts recently published by The Cancer Genome Atlas Research Network (TCGA) reported that 16% of colon cancers and up to 35% of endometrial cancers exhibit hypermutation[3,4]. For both colon and endometrial cancers, about three quarters of hypermutated tumors were associated with phenotypic microsatellite instability (MSI) and loss-of-function DNA mismatch repair genes via mutation or epigenetic silencing. Therefore, we hypothesized that hypermutated prostate cancer may also be associated with DNA mismatch repair (MMR) gene defects and MSI.

In this study we identified hypermutation in 7 of 60 patients with advanced prostate cancer. Using a targeted deep sequencing approach we find that all hypermutated tumors have somatic mutations in MMR genes and associated MSI. In four of seven hypermutated cases MMR mutations were complex structural rearrangements in *MSH2* and *MSH6*. We conclude that somatic rearrangements in *MSH2* and *MSH6* are an important mechanism leading to hypermutation and MSI in advanced prostate cancer.

Results

Prevalence of hypermutation. We identified hypermutated cases in exome sequencing data sets of advanced prostate cancer samples from two sources: a panel of patient-derived xenografts (PDX) and metastatic specimens obtained through a rapid autopsy programme (Supplementary Table 1). Exome data for PDX tumors was from Kumar *et al.*[1], where hypermutation was previously characterized. In the autopsy samples where hypermutation status had not been previously established, we defined hypermutation as >300 somatic protein altering mutations based on the distribution of total mutation burden in metastatic tumors, which had matched normal tissue available (Supplementary Fig. 1; Supplementary Table 1). We identified hypermutation in 3 of 15 PDX tumors (Table 1), and in metastatic tumors from 5 of 50 autopsy patients (Table 2). There was partial overlap between the two patient groups: five of the PDX tumors were derived from autopsy patients, including one with a hypermutated genome (LuCaP 147). Therefore, there were a total of 7/60 unique patients with hypermutated tumors, for an overall prevalence of 11.6%. Hypermutation status was 100% concordant at different metastatic sites, and was also concordant between primary tumour and metastasis in two patients where primary prostate tumors were available (Table 2).

Identification of *MSH2* and *MSH6* rearrangements. Because exome sequencing has limitations in detecting structural rearrangements and larger insertion/deletion (indel) mutations, we investigated alterations in DNA MMR pathway genes in hypermutated and non-hypermutated cases using a targeted deep sequencing approach (BROCA assay) that included capture of intronic and flanking DNA sequences (Supplementary Table 2)[5,6]. We developed a bioinformatics pipeline to accurately detect structural variation, copy number variation and indel mutations of all sizes[7].

All three PDX hypermutated tumors had complex structural rearrangements in *MSH2*, *MSH6* or both genes (Table 1; Fig. 1a; Supplementary Figs 2–4), while only 1 of 20 non-hypermutated xenografts had mutations in these genes (LuCaP 145, derived from a patient with neuroendocrine prostate cancer, Supplementary Fig. 5). A second loss-of-function mutation in *MSH2* or *MSH6* was detected in the three hypermutated PDX tumors, but not in LuCaP 145, supporting a requirement for bi-allelic gene inactivation underlying the hypermutated genome.

We detected mutations with predicted loss-of-function in *MSH2*, *MSH6* or both genes in four of five rapid autopsy patients

Table 1 | MMR gene mutations in prostate cancer PDX.

PDX tumour*	Patient-derived from	Hypermutated?†	MSI	MMR gene mutation(s)‡
LuCaP 58		Yes	Yes	(1) *MSH6* del exon 8 to 3′UTR (2) *MSH6* frameshift (c.3799_3800del)
LuCaP 73		Yes	Yes	(1) *MSH2* and *MSH6* copy loss (del 3 Mb) (2) *MSH2-FBXO11* inversion
LuCaP 147, 147CR	05-165	Yes	Yes	(1) *MSH2-C2orf61* 343 kb inversion (2) *MSH2-KCNK12* 74 kb inversion (3) *MSH2-KCNK12* 40 kb inversion
LuCaP 23.1, 23.1CR		No	No	None
LuCaP 35, 35CR		No	No	None
LuCaP 70, 70CR		No	No	None
LuCaP 77, 77CR		No	No	None
LuCaP 78	98-328	No	No	None
LuCaP 81	98-362	No	No	Chr2 copy losses
LuCaP 86.2, 86.2CR		No	No	None
LuCaP 92	99-069	No	No	None
LuCaP 96, 96CR		No	No	None
LuCaP 105, 105CR		No	No	None
LuCaP 141		No	No	None
LuCaP 145.1, 145.2	05-144	No	No	(1) *MSH2* exon 8-16 del (2) *MSH6-TESC* t(2;12)

MMR, mismatch repair; MSI, microsatellite instability; PDX, patient-derived xenografts.
*Matched pairs of androgen-sensitive and castration-resistant sublines (for example, LuCaP 35 and LuCaP 35CR) and tumour lines derived from the same patient are listed numerically and grouped in the same row.
†Hypermutation status was previously determined in these samples in Kumar *et al.*[1]
‡Mosaic *MSH6* frameshift mutations observed in a poly G tract in exon 5 (c.3261dup/del) and poly A tract in exon 7 (c.3573del) were detected in several hypermutated samples and are not included in the table because they are presumed to be due to MSI.

© 2014 Macmillan Publishers Limited. All rights reserved.

Table 2 | MMR gene mutations in rapid autopsy patients.

Autopsy patient*	Tumour site(s) tested by BROCA targeted sequencing	Mutation burden† (exome)	Hypermutated?	MSI	MMR gene mutation(s)‡
05-165*	Bone, adrenal, liver and lymph node	855	Yes	Yes	(1) *MSH2-C2orf61* 343 kb inversion (2) *MSH2-KCNK12* 74 kb inversion (3) *MSH2-KCNK12* 40 kb inversion
03-130	Lymph node	647	Yes	Yes	(1) *MSH2* translocation splits the gene t(2;18) (2) *MSH2* copy loss (3) *MSH6* frameshift (c.2690del) (4) *MSH6* copy loss
06-134	Kidney and lymph node	314	Yes	Yes	*MLH1* homozygous copy loss
00-010	Prostate and liver	673	Yes	Yes	*MSH2* frameshift (c.2364_2365insTACA)
05-123	Prostate and lymph node	807	Yes	Yes	(1) *MSH2* frameshift (c.1124_1125insG) (2) *MSH2* frameshift (c.1082del) (3) *MLH1* frameshift (c.1310del), lymph node only
01-095	Liver and lymph node	149	No	No	None
05-144*	Bone, adrenal, liver and lymph node	57	No	No	(1) *MSH2* exon 8-16 del (2) *MSH6-TESC* t(2;12)
05-214	Bone, liver and lymph node (two sites)	46	No	No	None
05-116	Bone, adrenal, liver and lung	47	No	No	None
00-029	Liver	37	No	No	None
00-090	Lymph node	69	No	No	None

MMR, mismatch repair; MSI, microsatellite instability.
*Fifty total unique autopsy patients were assessed by exome sequencing (see Supplementary Table 1). Listed are a subset of cases that were followed up by targeted deep sequencing for MMR genes. Clinical data for this patient subset is provided in Supplementary Table 6. Patient-matched non-cancer tissue was tested in every case and did not exhibit MSI or MMR mutations. LuCaP 147 and 147CR are derived from autopsy patient 05-165. LuCaP 145.1 and 145.2 are derived from autopsy patient 05-144.
†Number of protein altering somatic mutations by exome sequencing with removing of germline variants from matched-non-tumour samples.
‡Mutations were detected at every tumour site unless otherwise indicated. Mosaic *MSH6* frameshift mutations observed in a poly G tract in exon 5 (c.3261dup/del) and poly A tract in exon 7 (c.3573del) were detected in several hypermutated samples and are not included in the table because they are presumed to be due to MSI.

with hypermutated tumors. Mutations included complex structural rearrangements, copy losses and frameshift mutations (Table 2; Supplementary Figs 4 and 6–9). Two hypermutated patients had mutations in the MMR gene *MLH1*. We interrogated a subset of six non-hypermutated patients by deep sequencing and did not detect MMR gene mutations except in patient 05–144 from which the PDX LuCaP 145 was derived (Table 2). Like hypermutation status, MMR mutations were concordant at different metastatic sites in the same patient. MMR mutations were also concordant between primary tumour and metastasis except for a single *MLH1* frameshift mutation in patient 05–123 not found in the primary tumour (Table 2; Supplementary Fig. 9). Patient-matched non-tumour tissues were tested for the autopsy patients (Supplementary Table 1 and Supplementary Data 1). No MMR mutations were detected in patient-matched non-tumour tissue, indicating that none of the MMR mutations were inherited in the germline. Mutations in additional DNA repair genes are given in Supplementary Table 3.

Hypermutated tumors have phenotypic MSI. *MSH2* and *MSH6* are mismatch DNA repair genes that act together as a heterodimer, and bi-allelic inactivating mutations of either gene are predicted to result in MSI. PCR of microsatellite loci revealed MSI in all hypermutated tumors, from both PDX and autopsy patients (Fig. 1b; Supplementary Data 1). Phenotypic MSI was also detected directly from targeted next-generation data for all hypermutated tumors, and not detected in any non-hypermutated tumors (Supplementary Data 1; Supplementary Fig. 10). Immunohistochemistry (IHC) for DNA MMR proteins in hypermutated tumors demonstrated complete loss of MSH2 and/or MSH6 in a pattern consistent with the inactivating mutations detected by sequencing (Fig. 1c; Supplementary Fig. 11). Non-hypermutated tumors were microsatellite stable (Tables 1 and 2; Supplementary Data 1) and had intact MSH2 and MSH6 proteins, except LuCaP 145, which exhibited heterogeneous loss of MSH6 protein (Fig. 1c). *MLH1* methylation was not detected in any of the MSI positive tumors (Supplementary Fig. 12), and MLH1 protein expression was intact by IHC in MSI-positive tumors except in 06–134 that had homozygous *MLH1* gene deletion (Supplementary Fig. 13), arguing that *MLH1* epigenetic silencing was not responsible for MSI in any of the tumors in our series.

Discussion

Our findings support the conclusion that the hypermutated subtype of prostate cancer is chiefly due to loss-of-function mutations in *MSH2* and *MSH6* that result in MSI. Mutations were predicted to be bi-allelic in all cases except 00–010, which may harbour a second undetected mutation. Most interestingly, four of seven hypermutated cases had complex structural rearrangements in *MSH2* and *MSH6* that were not detected by exome sequencing in the same samples, and would also not be expected to be detected by traditional exon-based Sanger sequencing methods. Several previous studies have reported MMR protein loss and MSI in both primary and advanced prostate cancers, but very few MMR mutations have been identified[8–15]. We speculate that technical limitations have led to an underestimation of MMR gene mutations in prostate cancer.

Our finding of predominantly *MSH2* and *MSH6* mutations is in contrast to colon and endometrial cancer, where MSI is most often due to *MLH1* epigenetic silencing[3,4]. This supports an alternate mechanism by which MSI is acquired in prostate cancer. A recent study demonstrated that DNA translocations and deletions in advanced prostate cancer occur in a highly

© 2014 Macmillan Publishers Limited. All rights reserved.

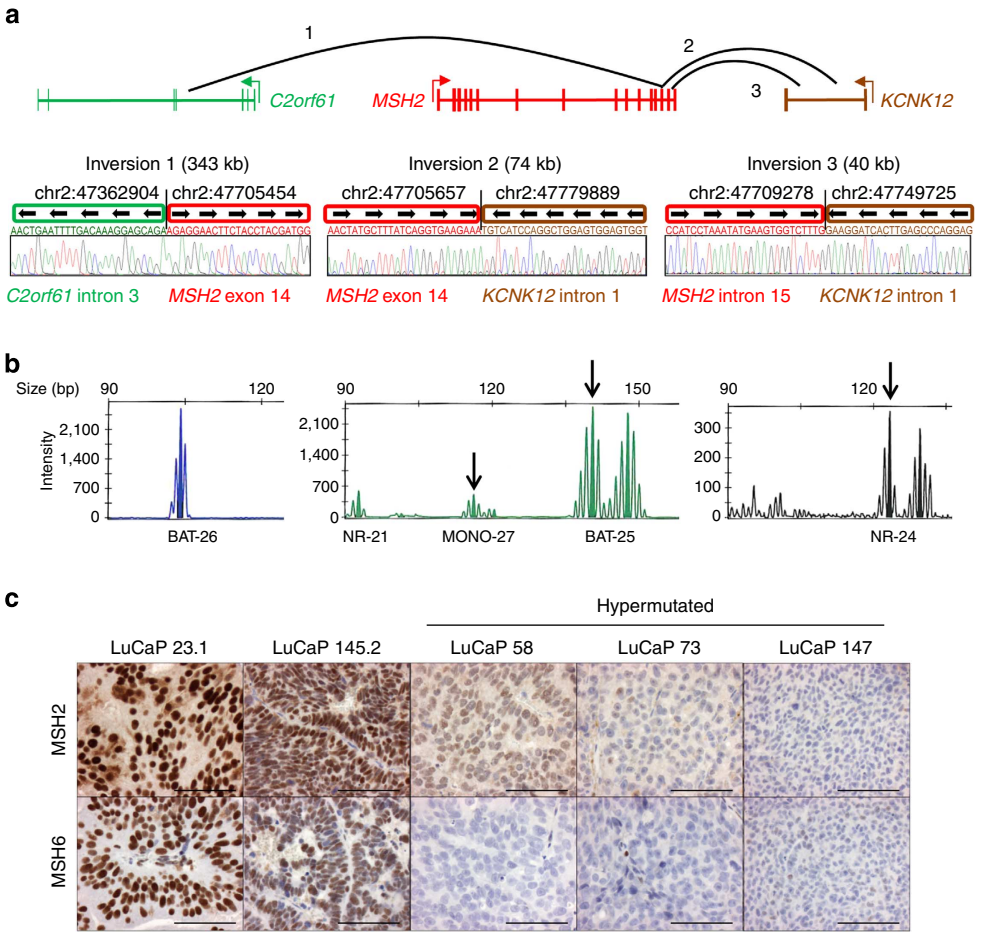


Figure 1 | *MSH2* and *MSH6* rearrangements are associated with loss of protein expression and MSI. (**a**) Four of seven hypermutated cases had complex rearrangements in *MSH2* and *MSH6* or both genes. Shown is a representative complex *MSH2* rearrangement present in hypermutated cases LuCaP 147 and 05-165 (LuCaP 147 was derived from autopsy patient 05-165). Breakpoints were confirmed by Sanger sequencing. Genomic coordinates are hg19. Detail on additional structural rearrangements and other mismatch repair gene mutations is provided in Tables 1 and 2 and Supplementary Figs 2–9. (**b**) Hypermutated tumors exhibited microsatellite instability by PCR. Shown is representative data for LuCaP 58, which is positive for MSI in 3/5 mononucleotide marker systems (MONO-27, BAT-25 and NR-24, arrows). All hypermutated tumors tested were MSI-PCR positive in at least 2/5 loci (Supplementary Data 1). (**c**) Hypermutated tumors LuCaP 58, 73 and 147 have loss of MSH2 and MSH6 proteins by IHC. Similar results were observed in hypermutated tumors from rapid autopsy patients (Supplementary Fig. 11). A representative non-hypermutated tumour (LuCaP 23.1) has intact expression. LuCaP 145 had mono-allelic mutations in *MSH2* and *MSH6* but was not hypermutated. IHC shows loss of MSH6 protein expression in some tumour cells. Scale bars, 0.1 mm.

interdependent manner, a process termed 'chromoplexy'[16]. This process may play a role in the genesis of *MSH2* and *MSH6* structural rearrangements and deserves future study. Androgen receptor (AR) function may also play a role in the formation of *MSH2* and *MSH6* structural alterations. AR has recently been implicated in the genesis of gene rearrangements in prostate cancer by facilitating double-strand DNA breaks and inducing non-homologous end-joining (reviewed in refs 17,18).

In summary, we have shown that complex structural rearrangements in mismatch DNA repair genes *MSH2* and *MSH6* are a major mechanism underlying hypermutation in advanced prostate cancer. Future studies should focus on determining if patients with MMR gene defects exhibit a distinct clinical course and are differentially responsive to genotoxic therapy.

Methods

Patients and specimens. The LuCaP series of prostate cancer xenografts were obtained from the University of Washington Prostate Cancer Biorepository.

Human primary and metastatic prostate cancer tissues were obtained as part of the University of Washington Prostate Cancer Donor Rapid Autopsy Programme. A haematoxylin and eosin slide was reviewed and scrolls from tissue blocks with >50% estimated tumour purity were used. The Institutional Review Board of the University of Washington approved all procedures involving human subjects, and all subjects signed written informed consent. The sample size was chosen based on the number of cases with suitable tissues for exome sequencing.

Genomic DNA was prepared from either formalin-fixed paraffin-embedded tissue or from fresh-frozen tissue (for bone metastases) with the Gentra Puregene DNA Isolation Kit (Qiagen, Catalogue #158489).

Immunohistochemistry. Expression of MMR proteins was determined by IHC using a tissue microarray (UWTMA55), that consisted of 155 metastatic prostate cancer sites from 50 patients, including 77 soft tissue metastases and 83 bone metastases), UWTMA52 consisting of primary prostate cancer obtained at the time of radical prostatectomy from 127 patients, and UWTMA 63 that consisted of prostate cancer tissue from 32 different LuCaP xenograft lines. All the tissue cores were duplicated.

Formalin-fixed paraffin-embedded tissue sections (5 μm) were deparaffinized and rehydrated with three changes of xylene and graded ethanol. Antigen retrieval was performed with heat-induced epitope retrieval for 20 min. Endogenous peroxide and avidin/biotin was blocked and sections were then blocked with 5% normal goat-horse-chicken serum at room temperature for 1 h, and incubated with primary antibody (listed in table below) at 4 °C overnight. After washing three times with 1 × PBS, slides were incubated with biotinylated secondary antibody (Vector Laboratories Inc.), followed by ABC reagent (Vector Laboratories Inc.)

© 2014 Macmillan Publishers Limited. All rights reserved.

and stable diaminobenzidine (Invitrogen Corp.). All sections were lightly counterstained with haematoxylin and mounted with Cytoseal XYL (Richard Allan Scientific). Mouse or rabbit immunoglobulin-G was used at the same concentration as the primary antibody for negative controls. Antibodies and dilutions used for IHC are given in Supplementary Table 4.

Immunostaining was assessed using a quasi-continuous score system, created by multiplying each intensity level ('0' for no brown colour, '1' for faint and fine brown chromogen deposition and '2' for clear and coarse granular chromogen clumps) with the corresponding percentage of cells expressing the particular intensity, and then summing all values to get a final score for each sample (scores ranging from 0 to 200). Only nuclear staining was evaluated. Samples with damaged tissue core, missing tissue core or poor quality of tissue were excluded from finial analysis.

Microsatellite instability PCR. MSI-PCR testing was performed by the University of Washington (UW) clinical genetics and solid tumors laboratory using the Promega MSI analysis kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Specimens demonstrating instability within two or more of the five mononucleotide markers included in this panel were considered 'MSI positive', others were considered 'MSI negative'. The microsatellite loci tested in the Promega MSI analysis kit were NR-21, BAT-26, BAT-25, NR-24 and MONO-27 (Genbank Accession # XM_033393, U41210, L04143, X60152, AC007684, respectively).

***MLH1* methylation analysis.** Two to four hundred nanograms of DNA from each sample was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) and eluted in 20 µl volume, according to manufacturer's protocol.

SYBR Green qPCR to detect methylated and unmethylated *MLH1* was performed using a CFX 96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with a final reaction volume of 20 µl, consisting of 500 nM each primer, 9 ng of bisulfite-converted genomic DNA and iTaq Universal SYBR Green Supermix at the following conditions: 95 °C for 3.5 min, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The unique primer sequences for methylated *MLH1* were 5′-CGGATAGCGATTTTTAACGC-3′ (forward) and 5′-CCTAAAACGACTACTACCCG-3′ (reverse), and for unmethylated *MLH1* were 5′-AATGAATTAATAGGAAGAGTGGATAGT-3′ (forward) and 5′-TCTCT TCATCCCTCCCTAAAACA-3′ (reverse) (ref. 19). The four primers each also included a 20 bp GC-rich tail (5′-GCGGTCCCAAAAGGGTCAGT-3′) at their 5′ end. Repetitive Alu sequence ('AluC4') was used to normalize for the amount of input DNA2. The absolute quantitation of methylated and unmethylated *MLH1* in each sample was determined by using the Epitect human methylated and unmethylated DNA (Qiagen, Germantown, MD, USA) to create a standard curve. The SYBR Green assay results are expressed as ratios between methyl-*MLH1* or unmethyl-*MLH1* values and the ALUC4 control values. The error bars represent the s.e.m.

Exome sequencing. Exome sequencing for autopsy samples was performed using the Nimblegen EZ SeqCap kit (Roche)[1,20]. Shotgun libraries were constructed by shearing DNA and ligating sequencing adaptors. Libraries were hybridized to either the EZSeqCap V1 or V2 solution-based probe, amplified and sequenced on either the Illumina GAIIx or HiSeq platform. For all metastases, somatic mutations were called using Mutect using default parameters with matched normal (non-tumour) samples. To remove common polymorphisms and other artifacts, we imposed a number of additional requirements, including requiring variants to be observed with a variant allele fraction of at least 10% within a tumour, removing variants present within dbSNP v137 that had first been stripped of all disease-associated variants and removing variants that were present at an allele balance of 40% or more in any germline sample. All exome sequencing was performed on fresh-frozen tissue samples.

Exome data for PDX samples was from Kumar *et al.*[1], where hypermutation status was previously characterized based on the distribution of mutations across samples. For the xenografts, because corresponding normal germline DNA was not available, tumour sequences were compared against a database of common germline variants. The variants remaining were termed novel single-nucleotide variants SNVs ('novSNV') and the estimated the contribution of germline variants was ~200 and sometimes more per individual. novSNV counts from Kumar *et al.*[1] are provided in Supplementary Table 1.

Targeted deep sequencing by BROCA. Targeted deep sequencing of DNA repair pathway genes was performed using the BROCA assay in the UW clinical genetics and solid tumors laboratory[5]. Three micrograms of DNA was sonicated to a peak of 200 bp on a Covaris S2 instrument (Covaris, Woburn, MA, USA). Following sonication, DNA was purified with AMPure XP beads (Beckman Coulter, Brea CA, USA) and subjected to three enzymatic steps: end repair, A-tailing and ligation to Illumina paired-end adaptors as described in the SureSelectXT Target Enrichment for Illumina multiplexed sequencing, which is available for free download. Adapter-ligated library was PCR amplified for five cycles with Illumina primers 1.0 and 2.0 and individual paired-end libraries (500 ng) were hybridized to a custom design of complementary RNA biotinylated oligonucleotides targeting 53 genes in 52 genomic regions (Supplementary Table 2). The 120-mer oligonucleotide baits were designed in Agilent's eArray web portal with the following parameters: centred tiling, 3 × bait overlap and a maximum overlap of 20 bp into repetitive regions. The custom design targets a total of 1.4 Mb of DNA. Following capture, each library was PCR amplified for 13 cycles with primers containing a unique 6 bp index. Equimolar concentrations of 96 libraries were pooled to a final concentration of 10 pM, denatured with 3 N NaOH, and cluster amplified with a cBot instrument on a single lane of an Illumina v3 flowcell. Sequencing was performed with 2 × 101 bp paired-end reads and a 7 bp index read using SBS v3 chemistry on a HiSeq2500 (Illumina, SanDiego, CA, USA).

We used our targeted tumour sequencing bioinformatics pipeline for data analysis[21]. Reads were mapped to human reference genome (hg19/GRCh37) and alignment performed using BWA v0.6.1-r10419 and SAMtools v0.1.1820. SNV and indel calling was performed through the GATK Universal Genotyper using default parameters and using VarScan v2.3.2 and PINDEL version 0.2.42. Structural variants were identified using CREST v1.0 and BreakDancer v1.1. For copy number variant (CNV) analysis, copy number states for individual probes were initially called using CONTRA v2.0.32 with reference to a CNV control comprised of reads from two independent rounds of library preparation and sequencing of HapMap individual NA12878. CNV calls were made at the resolution of individual exons using custom Perl scripts. CNV plots were visualized using the R package ggplot2.

Phenotypic MSI was assessed directly from BROCA next-generation sequencing data using mSINGS (MSI by NGS)[22]. This method evaluated up to 146 mononucleotide microsatellite loci that are captured by BROCA in both matched normal non-tumour and tumour samples. For each specimen, microsatellite loci covered by a read depth of < 30 × were excluded as not passing quality filter. For each microsatellite locus passing quality filter, the distribution of size lengths were compared with a population of normal controls. Loci were considered unstable if the number of repeats is statistically greater than in the control population. A fraction of > 0.20 (20% unstable loci) was considered MSI-high by mSINGS based on validation with 324 tumour specimens, in which 108 cases had MSI-PCR data available as a gold standard[22].

Confirmation of *MSH2* and *MSH6* structural rearrangements. To validate structural rearrangement calls, we designed primers against regions flanking putative breakpoints using either PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm). We used the iProof High-Fidelity PCR kit (Bio-Rad) to perform PCR under the following conditions: 98 °C for 35 s followed by 30–40 cycles of 55–69 °C for 30 s, 72 °C for 30 s and 72 °C for 10 min. Primers are listed in Supplementary Table 5. We submitted resulting PCR products to Genewiz for Sanger sequencing and aligned fragments to the human genome reference sequence (hg19) using BLAT from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway).

Copy number changes were confirmed by genomic microarray. One microgram of high molecular weight genomic DNA from each sample was labelled by random priming using the Agilent Genomic DNA Enzymatic Labelling Kit (Cy3-dUTP.) A pool of reference normal DNA (Promega) was labelled with Cy5-dUTP. Cy3 and Cy5 probes were combined and hybridized to Agilent 2 × 400K SurePrint G3 CGH Microarrays and washed following the manufacturer's specifications. Fluorescent array images were collected using the Agilent DNA microarray scanner G2505C and Agilent Feature Extraction software. Data analysis was performed with Biodiscovery Nexus Copy Number 6.0 software. The FASST2 segmentation algorithm and default Agilent settings for significance, gain and loss thresholds, with at least six probes per segment were used to identify regions of CNV for each sample. Results of copy number analysis by genomic microarray are given in Supplementary Fig. 14.

References

1. Kumar, A. *et al.* Exome sequencing identifies a spectrum of mutation frequencies in advanced and lethal prostate cancers. *Proc. Natl Acad. Sci. USA* **108,** 17087–17092 (2011).
2. Barbieri, C. E. *et al.* Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. *Nat. Genet.* **44,** 685–689 (2012).
3. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **487,** 330–337 (2012).
4. Kandoth, C. *et al.* Integrated genomic characterization of endometrial carcinoma. *Nature* **497,** 67–73 (2013).
5. Pritchard, C. C. *et al.* ColoSeq provides comprehensive lynch and polyposis syndrome mutational analysis using massively parallel sequencing. *J. Mol. Diagn.* **14,** 357–366 (2012).
6. Walsh, T. *et al.* Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing. *Proc. Natl Acad. Sci. USA* **107,** 12629–12633 (2010).
7. Pritchard, C. C. *et al.* Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. *J. Mol. Diagn.* **16,** 56–67 (2014).

© 2014 Macmillan Publishers Limited. All rights reserved.

8. Jarzen, J., Diamanduros, A. & Scarpinato, K. D. Mismatch repair proteins in recurrent prostate cancer. *Adv. Clin. Chem.* **60,** 65–84 (2013).
9. Burger, M. *et al.* Elevated microsatellite instability at selected tetranucleotide repeats does not correlate with clinicopathologic features of bladder cancer. *Eur. Urol.* **50,** 770–775 (2006).
10. Chen, Y. *et al.* Defects of DNA mismatch repair in human prostate cancer. *Cancer Res.* **61,** 4112–4121 (2001).
11. Velasco, A. *et al.* Differential expression of the mismatch repair gene hMSH2 in malignant prostate tissue is associated with cancer recurrence. *Cancer* **94,** 690–699 (2002).
12. Sun, X., Chen, C., Vessella, R. L. & Dong, J. T. Microsatellite instability and mismatch repair target gene mutations in cell lines and xenografts of prostate cancer. *Prostate* **66,** 660–666 (2006).
13. Chen, Y. *et al.* Alterations in PMS2, MSH2 and MLH1 expression in human prostate cancer. *Int. J. Oncol.* **22,** 1033–1043 (2003).
14. Dahiya, R. *et al.* High frequency of genetic instability of microsatellites in human prostatic adenocarcinoma. *Int. J. Cancer* **72,** 762–767 (1997).
15. Watanabe, M. *et al.* Microsatellite instability in human prostate cancer. *Br. J. Cancer* **72,** 562–564 (1995).
16. Baca, S. C. *et al.* Punctuated evolution of prostate cancer genomes. *Cell* **153,** 666–677 (2013).
17. White, N. M., Feng, F. Y. & Maher, C. A. Recurrent rearrangements in prostate cancer: causes and therapeutic potential. *Curr. Drug Targets* **14,** 450–459 (2013).
18. Wu, D., Zhang, C., Shen, Y., Nephew, K. P. & Wang, Q. Androgen receptor-driven chromatin looping in prostate cancer. *Trends Endocrinol. Metab.* **22,** 474–480 (2011).
19. Petko, Z. *et al.* Aberrantly methylated CDKN2A, MGMT, and MLH1 in colon polyps and in fecal DNA from patients with colorectal polyps. *Clin. Cancer Res.* **11,** 1203–1209 (2005).
20. O'Roak, B. J. *et al.* Exome sequencing in sporadic autism spectrum disorders identifies severe de novo mutations. *Nat. Genet.* **43,** 585–589 (2011).
21. Pritchard, C. C. *et al.* Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. *J. Mol. Diagn.* **16,** 56–67 (2014).
22. Salipante, S. J., Scroggins, S. M., Hampel, H. L., Turner, E. H. & Pritchard, C. C. Microsatellite instability detection by next-generation sequencing. *Clin. Chem.* 24987110 (2014).

Acknowledgements

We thank Karen Koehler and Tatyana Marushchak for help with library preparation and sequencing. We thank Deborah Barden, Rachel Slusher, Laura Akagi and Youly Welt for their help in preparing the genomic DNA and with MSI-PCR. We thank Emily Turner for help with data analysis. We thank the University of Washington Rapid Autopsy team members and most importantly the patients and families who participated in research studies. This work was supported by awards P50CA097186, CA085859 and CA163227 from the National Cancer Institute, CDMRP awards PC131820 and PC093372P1 and a 2013 Young Investigator Award from the Prostate Cancer Foundation.

Author contributions

C.C.P. conceived and designed the study, coordinated sample acquisition and processing and performed primary data analyses. C.M., A.K. and P.S.N. assisted with the study design. T.W., J.S., R.L.V., E.C. and J.F.T. assisted with the study design and reviewed the manuscript. R.L.V., C.M. and E.C. were involved in metastasis and PDX tissues collection and selection. A.K., J.M. and S.J.S. performed confirmatory Sanger sequencing studies. C.M. and X.Z. performed and analyzed the IHC studies. C.S., I.C. and A.K. assisted with the genomic sequencing. I.C., S.J.S., C.C.P. and A.K. coordinated informatics analyses. W.M.G. and M.Y. performed *MLH1* methylation studies. C.C.P., P.S.N., R.L.V., T.W. and J.S. directed the research. C.C.P. wrote the manuscript, with contributions from P.S.N., A.K. and C.M.

Additional information

Accession codes: Sequencing data reported in this manuscript have been deposited in GenBank/EMBL/DDBJ under the accession code SRP044943.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

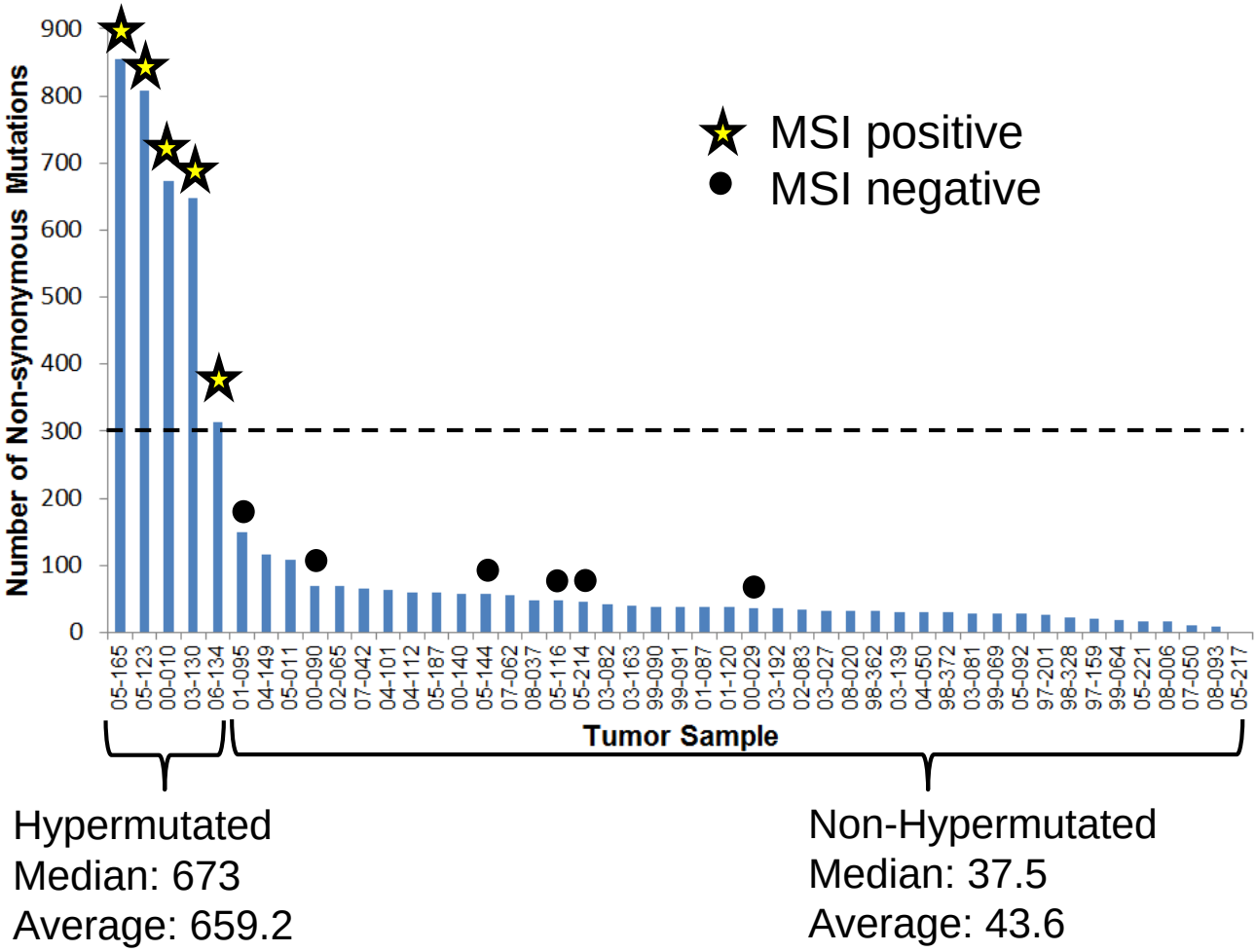
Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Pritchard, C. C. *et al.* Complex *MSH2* and *MSH6* mutations in hypermutated microsatellite unstable advanced prostate cancer. *Nat. Commun.* 5:4988 doi: 10.1038/ncomms5988 (2014).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© 2014 Macmillan Publishers Limited. All rights reserved.

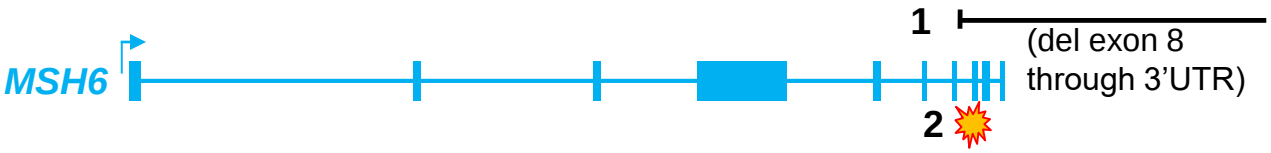


Supplementary Figure 1: Somatic Mutation Burden in Autopsy Cases by Exome Sequencing. Total number of somatic nonsynonymous mutations by exome sequencing for rapid autopsy cases. The threshold of 300 mutations used to determine hypermutation status is shown with the dashed line. Median and average mutation burden is given for both groups. Cases that had microsatellite instability testing are shown with yellow stars (positive) and black circles (negative).

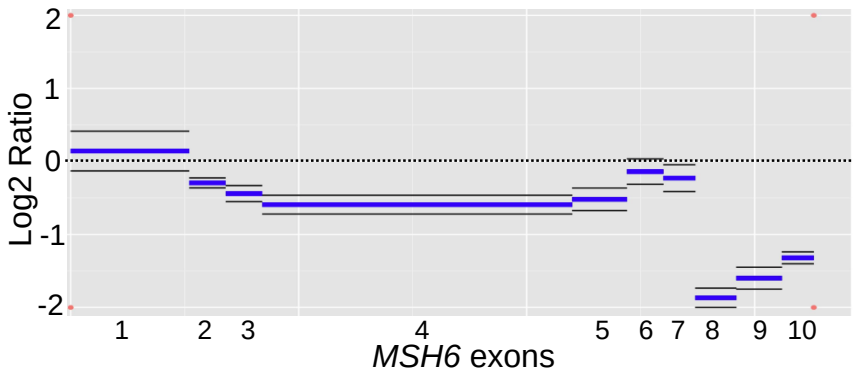
LuCaP 58

1) *MSH6* del exon 8 through 3'UTR

2) *MSH6* frameshift (c.3799_3800del)

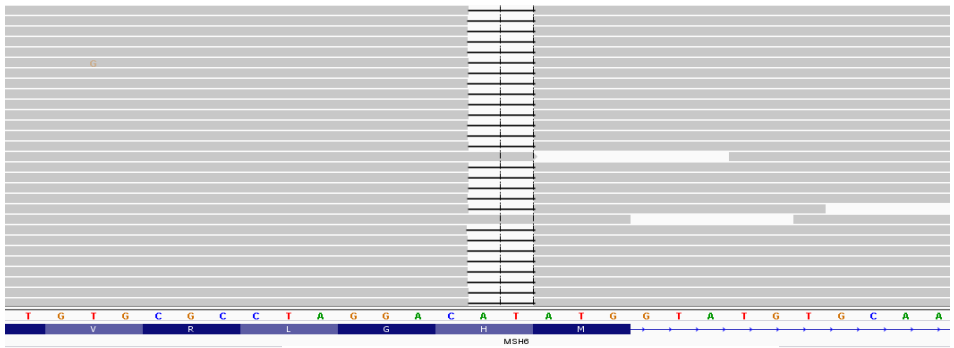


1)



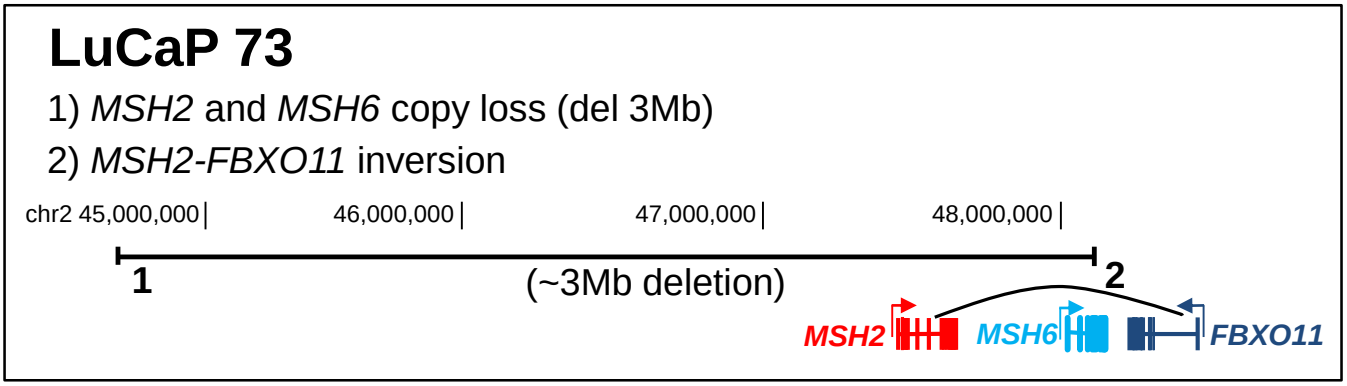
Deletion of exon 8 through 3'UTR

2) *MSH6* c.3799_3800del, p.M1267Gfs*7

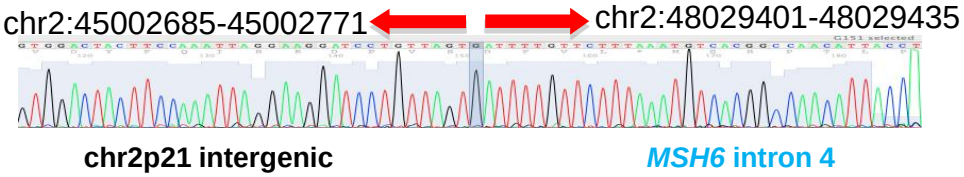


chr2:48033493-48033494delAT

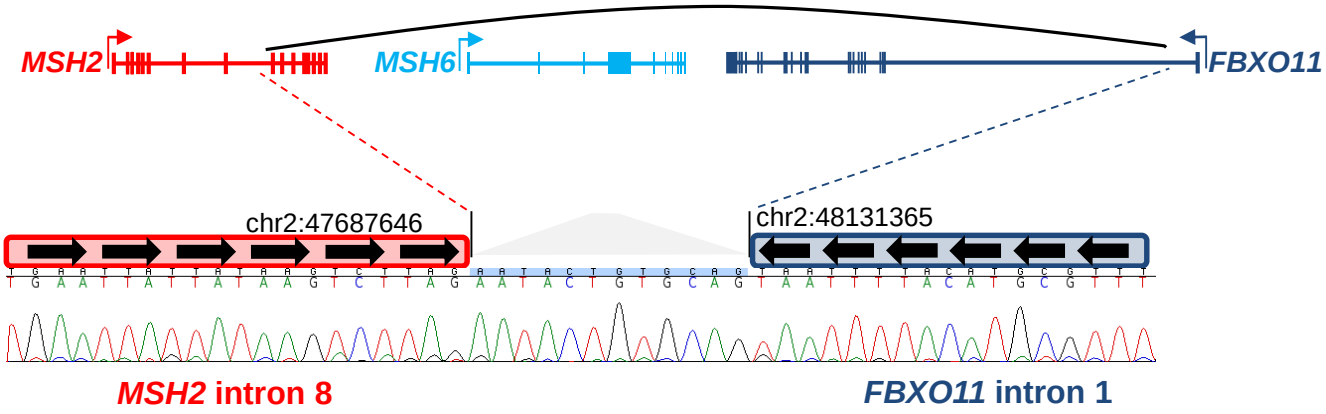
Supplementary Figure 2: Detail on Mismatch Repair Gene Mutations in LuCaP 58. Two inactivating mutations were detected in *MSH6*. The first (1) is a deletion of exon 8 through the 3'UTR (top). Copy number was calculated from normalized depth of coverage of BROCA sequencing data and confirmed by genomic microarray (data not shown). The blue bars indicate exons 1-10 (from left to right) and black bars are the standard deviation of the measurement of Log2 ratio. The second (2) is a 2bp deletion resulting in a frameshift and premature truncation of the MSH6 protein (c.3799_3800del, bottom). Shown is a screenshot from the integrated genomics viewer of representative sequencing reads. The black bars indicated the deleted bases. The frameshift was detected in 314 out of a total of 360 sequencing reads, strongly supporting that there is bi-allelic inactivation of *MSH6*.



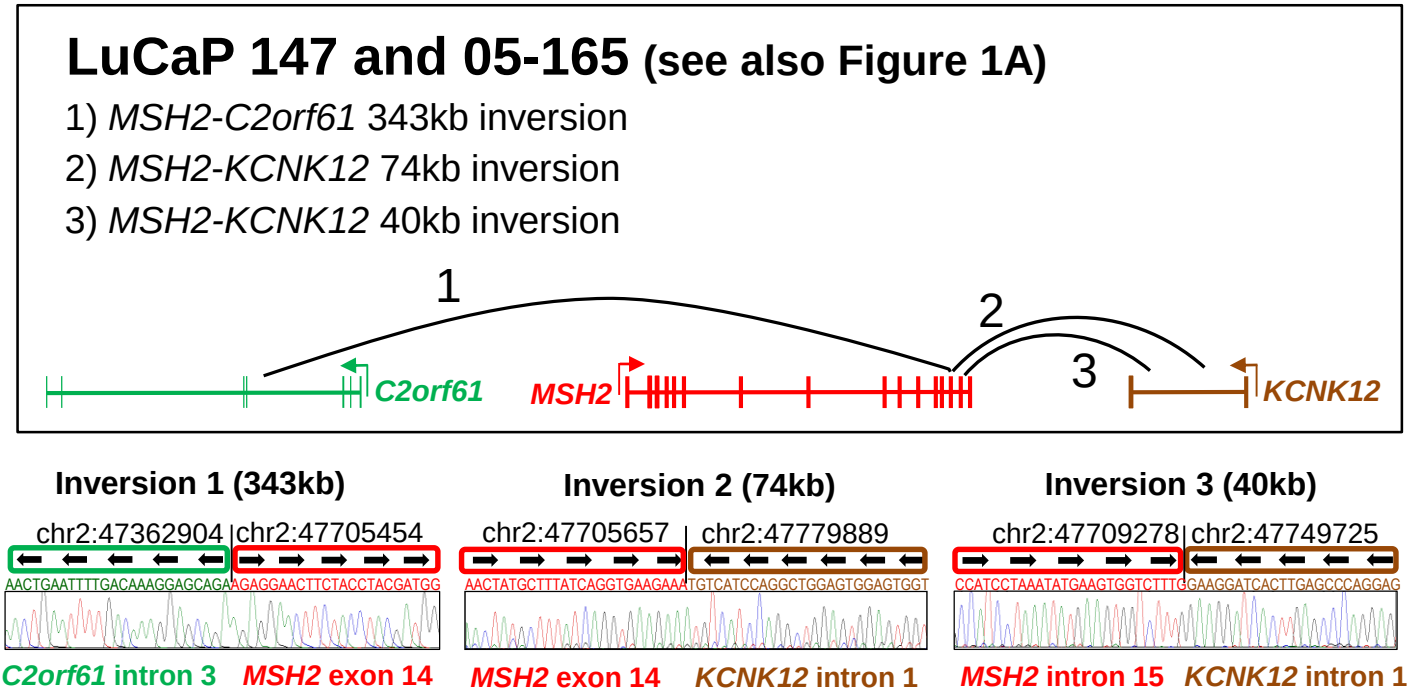
1) ~3MB deletion deletes *MSH2* and most of *MSH6*



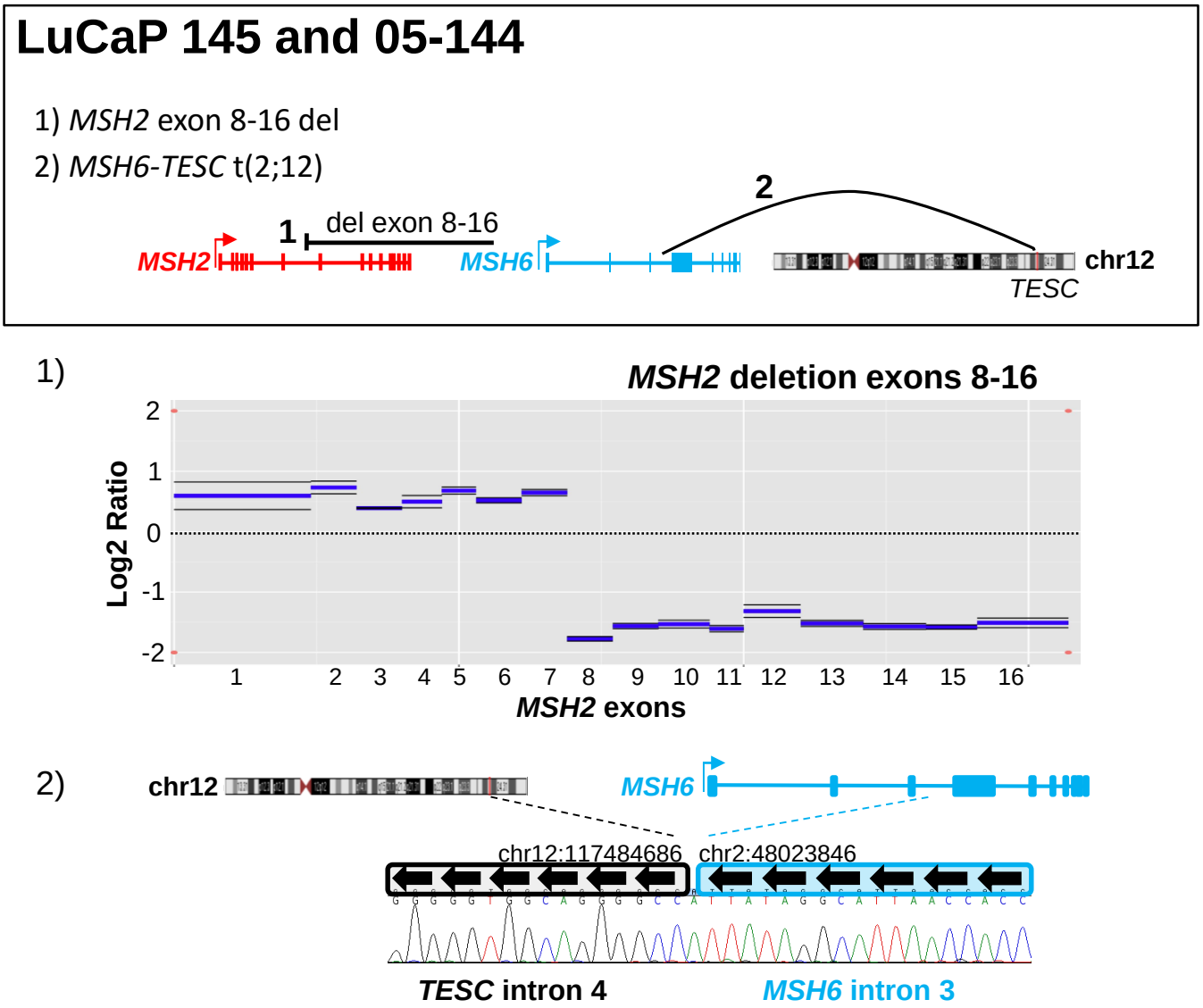
2) 440kb inversion splits the *MSH2* gene



Supplementary Figure 3: Detail on Mismatch Repair Gene Mutations in LuCaP 73. Two large rearrangement mutations were detected at the *MSH2/MSH6* locus on chromosome 2 predicted to result in bi-allelic inactivation of *MSH2*. The first (1) is a 3Mb deletion that deletes both the MSH2 and MSH6 genes. The breakpoints where confirmed by Sanger sequencing as chr2:45,002,771-48,029,401 in hg19 genomic coordinates (top). The second (2) is a 440kb inversion mutation between *MSH2* intron 8 and *FBXO11* intron 1 that splits the *MSH2* gene and is predicted to result in loss of function. The breakpoints of the inversion were confirmed by Sanger sequencing as chr2:47687644-chr2:48131365 (bottom). There is a short inserted sequence between the two breakpoints.

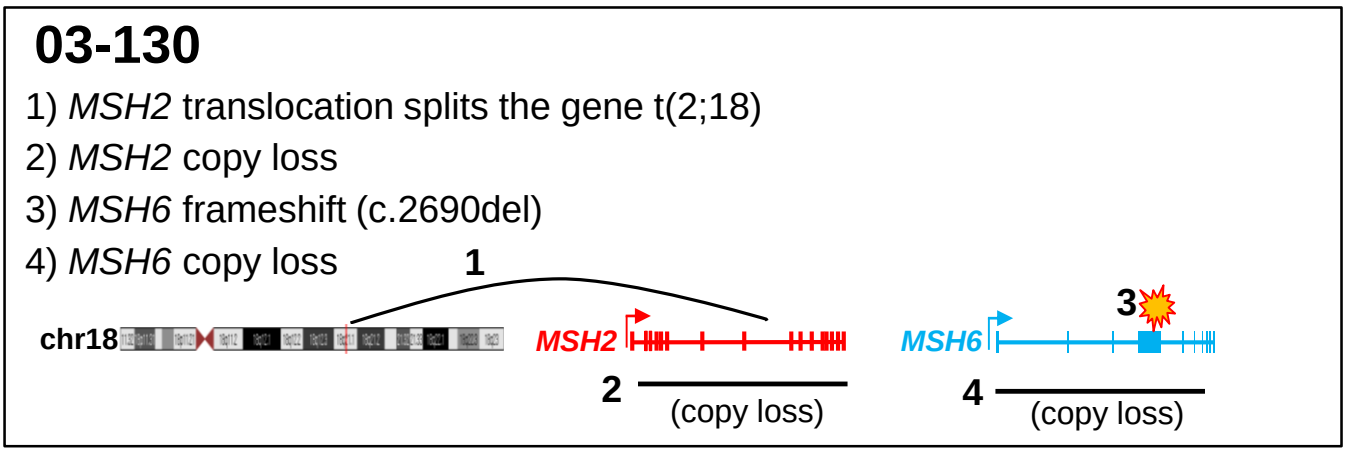


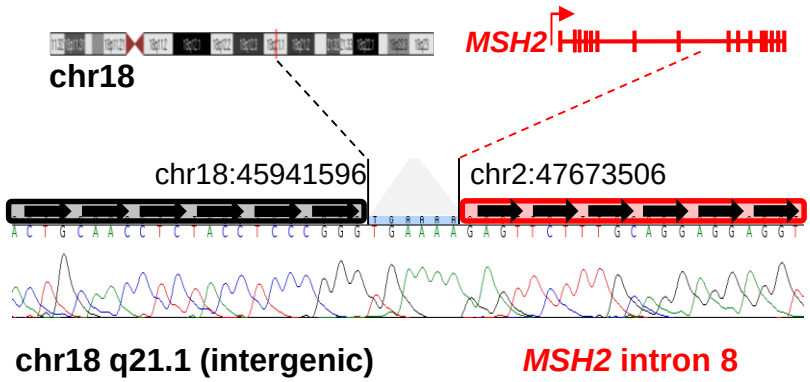
Supplementary Figure 4: Detail on Mismatch Repair Gene Mutations in LuCaP 147 and 05-165. Three different inversion mutations were detected involving the *MSH2* gene that are predicted to result in loss-of-function. The inversions were detected in all metastatic sites (bone, adrenal, liver, and lymph node). Each inversion was confirmed by Sanger sequencing with breakpoints given in hg19 genomic coordinates. LuCaP 147 was derived from autopsy patient 05-165 and the same mutations were detected in both, indicating that the *MSH2* structural rearrangements are not a result of xenografting.

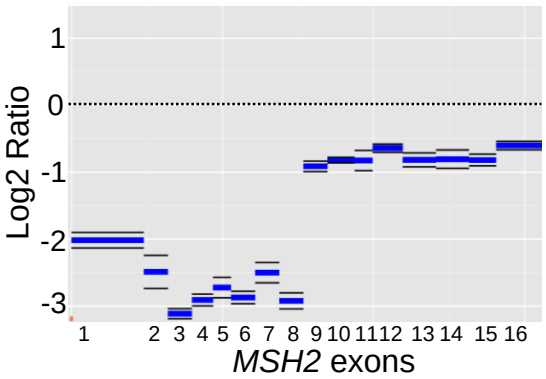


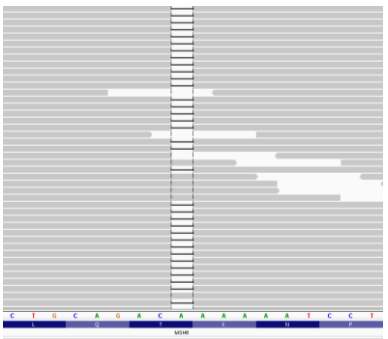
Supplementary Figure 5: Detail on Mismatch Repair Gene Mutations in LuCaP 145 and 05-144. Two mutations were detected. The first (1) is copy loss of exons 8-16 of *MSH2* (top). Copy number was calculated from normalized depth of coverage of BROCA sequencing data and confirmed by genomic microarray (data not shown). The blue bars indicate *MSH2* exons 1-16 (from left to right) and black bars are the standard deviation of the measurement of Log2 ratio. The second (2) is a translocation between *MSH6* intron 3 and *TESC* intron 4 on chromosome 12 q24.22. The breakpoints of the translocation were confirmed by Sanger sequencing. These tumors had neuroendocrine differentiation. Unlike the other tumors *MSH2/MSH6* rearrangements these tumors were not hypermutated and did not demonstrate MSI, most likely because one copy of *MSH2* and *MSH6* remain functionally intact. One hypothesis is that the cancer was 'transitioning' to a hypermutated state when the patient died, with a second hit in the *MSH2* or *MSH6* gene not yet acquired. LuCaP 145 was derived from autopsy patient 05-144 and the same mutations were detected in both, indicating that the structural rearrangements are not a result of xenografting.

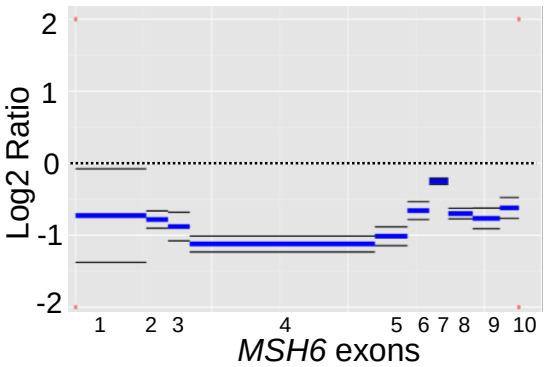
Supplementary Figure 6







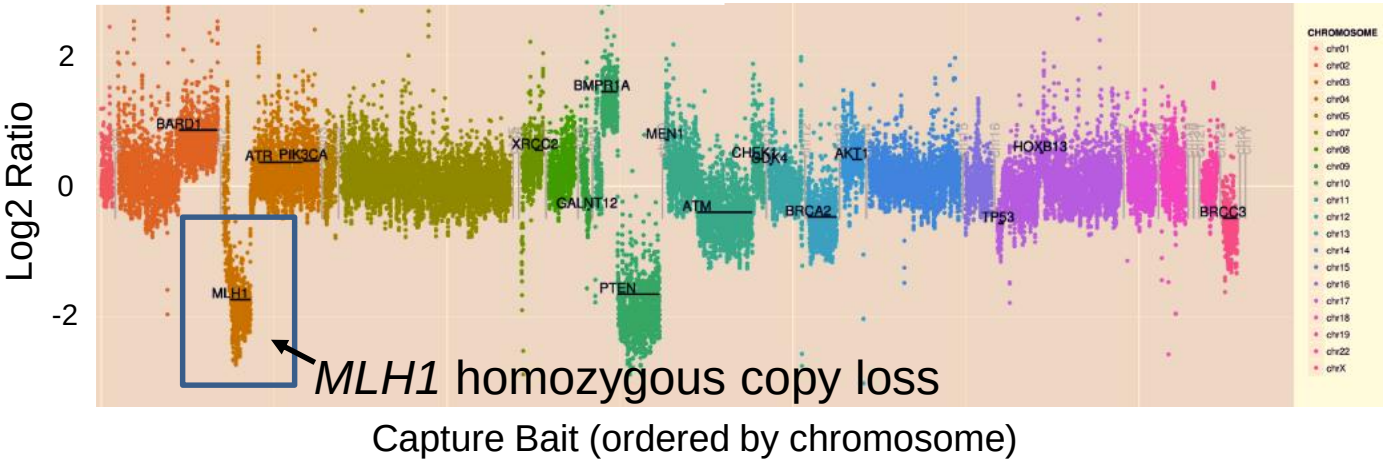




Supplementary Figure 6: Detail on Mismatch Repair Gene Mutations in 03-130. There was evidence of bi-allelic loss-of-function mutations in *MSH2* and *MSH6*. The first mutation (1) is a translocation between *MSH2* intron 8 *and* chr18 q21.1 (top left), in which the breakpoints were confirmed by Sanger sequencing. The second (2) is copy loss of *MSH2* (top right, homozygous in exons 1-8, likely as a result of the *MSH2* translocation). The third (3) is frameshift mutation in exon 4 of MSH6 (c.2690del, p.N897IfsX9). The black bars indicated the deleted bases. The frameshift was detected in 366 out of a total of 547 sequencing reads (67%), despite admixture of tumor with normal cells in the sample tested, supporting that there is bi-allelic inactivation of *MSH6* in tumor. The fourth (4) is copy loss of *MSH6*, probably single copy. In copy number plots blue bars indicate exons and black bars are the standard deviation of the measurement of Log2 ratio. Copy number was calculated by normalized depth of coverage of BROCA sequencing and confirmed by genomic microarray (data not shown)

06-134

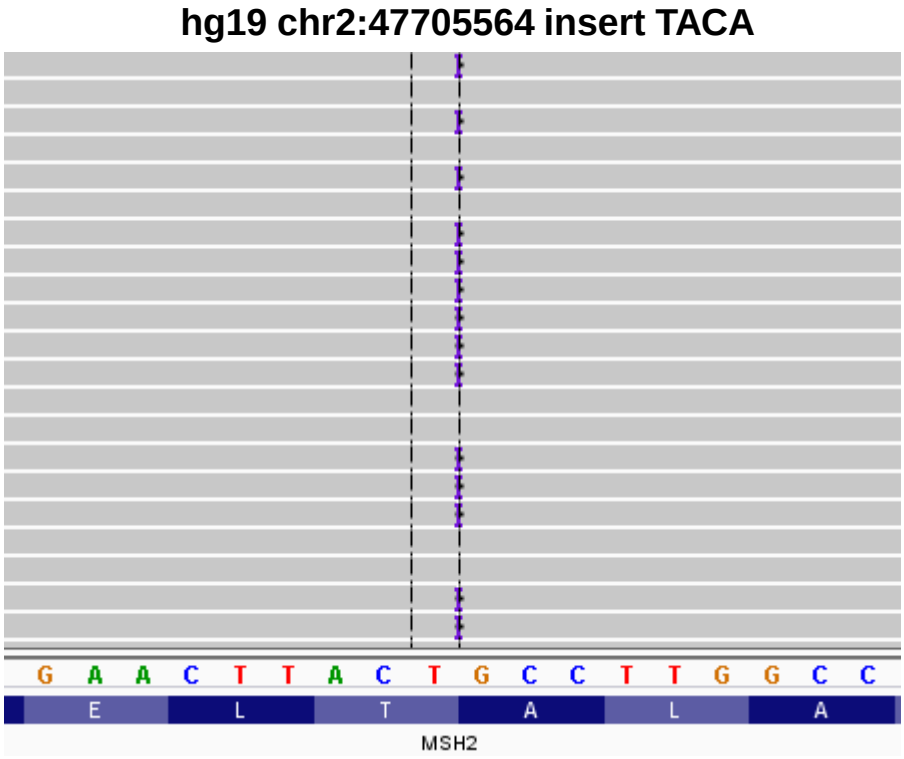
MLH1 homozygous copy loss



Supplementary Figure 7: Detail on Mismatch Repair Gene Mutations in 06-134. The sample tested had *MLH1* copy loss which is very likely to be homozygous and result in complete loss of MLH1 protein function. Depicted is copy number analysis by BROCA targeted deep sequencing. *MLH1* deletion was confirmed by genomic microarray (data not shown). The Log2 ratio in relationship to a normal female control patient. *BRCC3* (far right) on the X chromosome can be used to calibrate the Log2 ratio expected with heterozygous copy loss because this is a male patient. *PTEN* is also deleted in this patient's tumor, a common event in metastatic prostate cancer.

00-010

MSH2 frameshift (c.2364_2365insTACA, p.A789YfsX11)



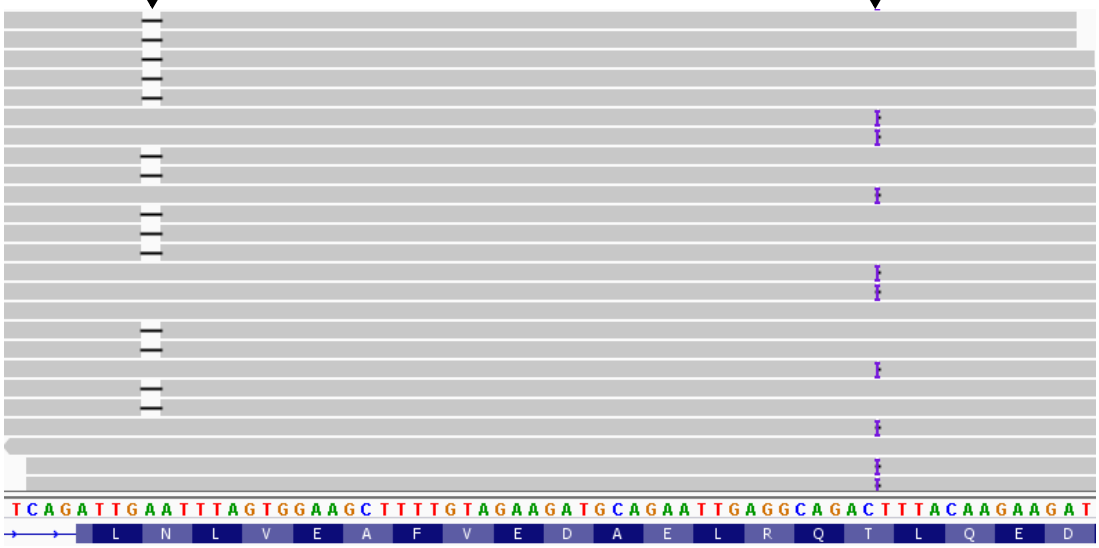
Supplementary Figure 8: Detail on Mismatch Repair Gene Mutations in 00-010. The primary prostate and liver tumor metastasis samples tested had a frameshift loss-of-function mutation in *MSH2* (c.2364_2365insTACA, p.A789YfsX11). The purple "I" indicates the position of the inserted bases, visualized in the integrated genomics viewer. A second loss-of-function mutation was not detected. It is suspected a second *MSH2* loss-of-function mutation is present because the case was MSI high, had loss of MSH2 and MSH6 protein by IHC, was *MLH1* unmethylated, and had intact MLH1 protein IHC (see separate figures).

05-0123

1) *MSH2* frameshift (c.1124_1125insG)
2) *MSH2* frameshift (c.1082del)
3) *MLH1* frameshift (c.1310del), lymph node only

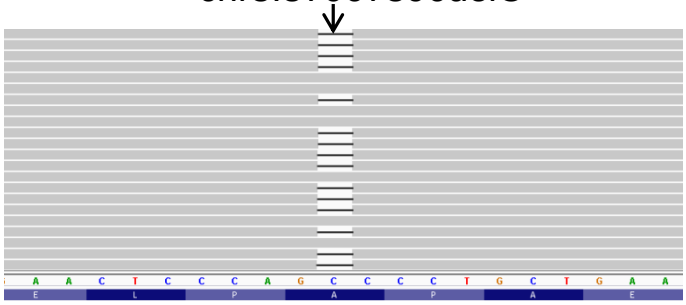
MSH2 c.1082del, p.N361IfsX2
chr2:47656885delA

MSH2 c.1124_1125insG, p.L376FfsX13
chr2:47656928insG



MSH2 exon 7

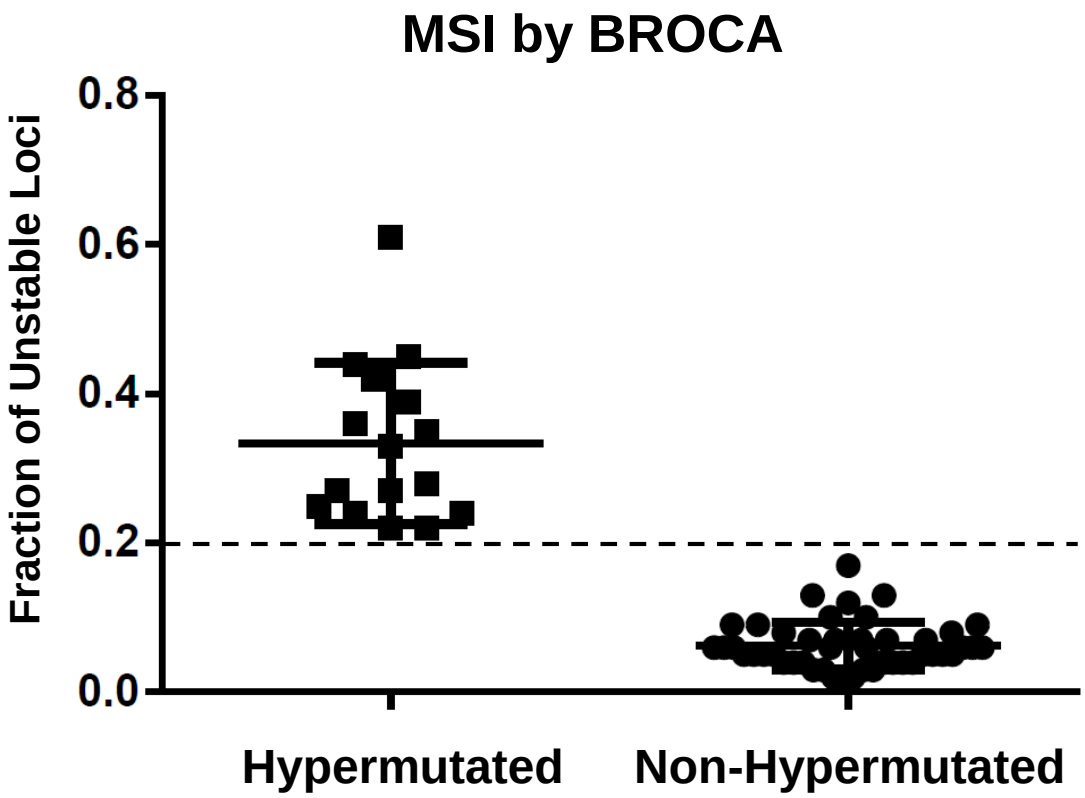
chr3:37067396delC



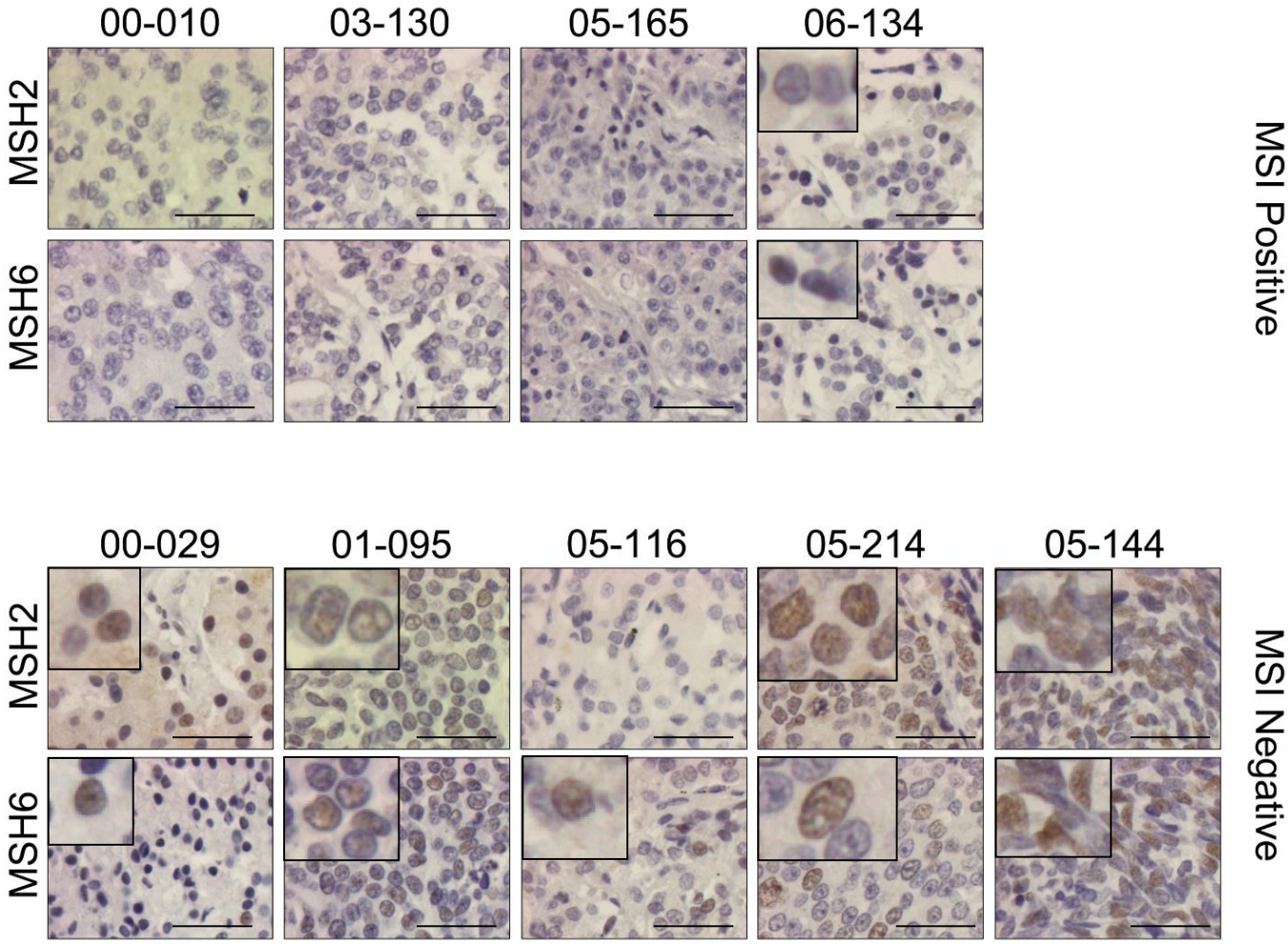
MLH1 exon 12

MLH1 c.1310del, p.P437LfsX54
(Lymph node metastasis only)

Supplementary Figure 9: Detail on Mismatch Repair Gene Mutations in 05-123. The primary prostate and lymph node metastasis tumor samples tested had bi-allelic frameshift loss-of-function mutations in *MSH2* exon 7 (c.1082del and c.1124_1125insG, top). The black bars indicate sequencing reads with the c.1082del deletion; the purple "I" indicates the position of the inserted bases in the c.1124_1125insertion, visualized in the integrated genomics viewer. Note that these two mutations do not occur on sequencing reads from the same allele, strongly supporting that they are *in trans (*bi-allelic). In addition, an *MLH1* frameshift mutation in exon 12 (c.1310delC) was detected in the lymph node metastasis sample only (bottom).

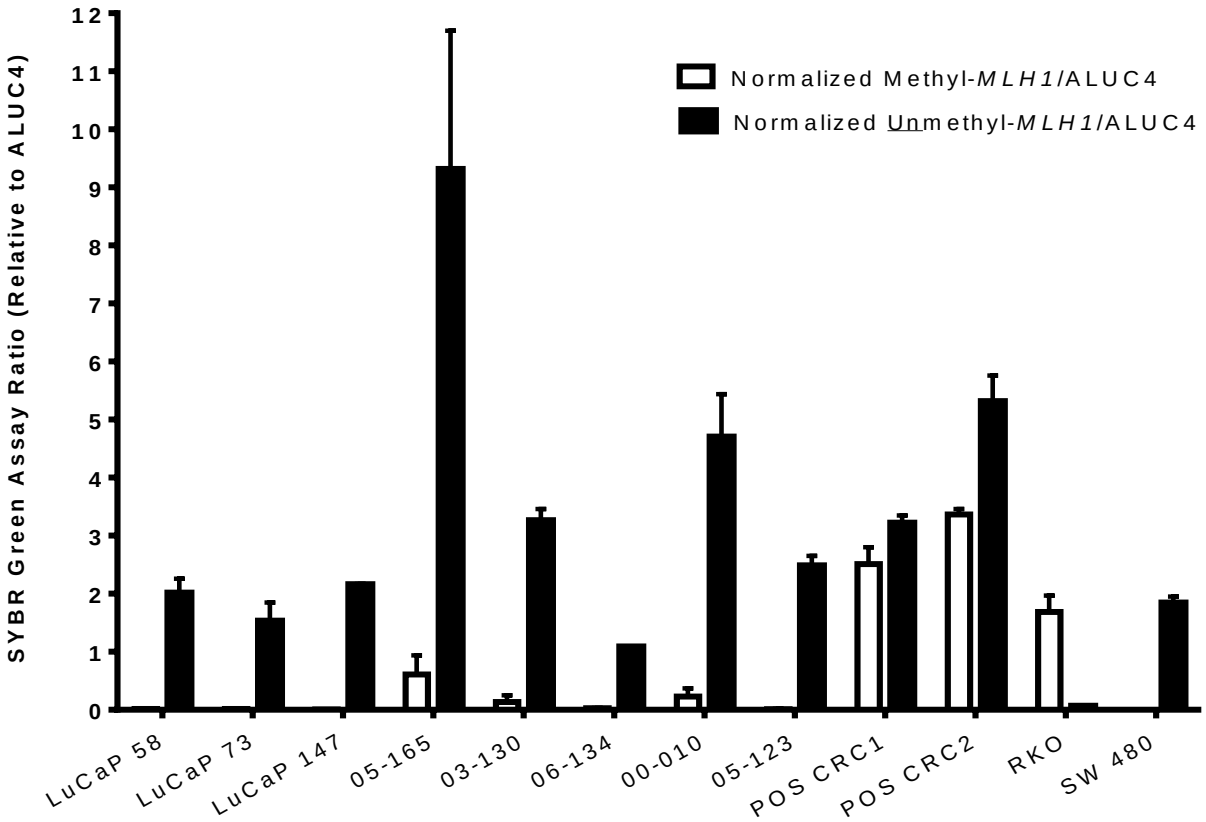


Supplementary Figure 10: Microsatellite Instability Results by BROCA Next-Generation Sequencing. We developed an approach to measure microsatellite instability directly from next-generation sequencing data that we call mSINGS. This method is described in the methods section (Salipante et al. 2014 *Clinical Chemistry*, in press). The fraction of unstable microsatellite loci out of a maximum of 146 mononucleotide microsatellite loci captured by BROCA is given on the y-axis. A threshold of 0.2 (20%) unstable loci was established as a cutoff for microsatellite instability (dashed line). Solid lines represent the median fraction of unstable loci for hypermutated and non-hyermutated cases. The raw data used to generate this summary figure, including which loci were unstable by BROCA in each sample is given in Supplementary Data 3.



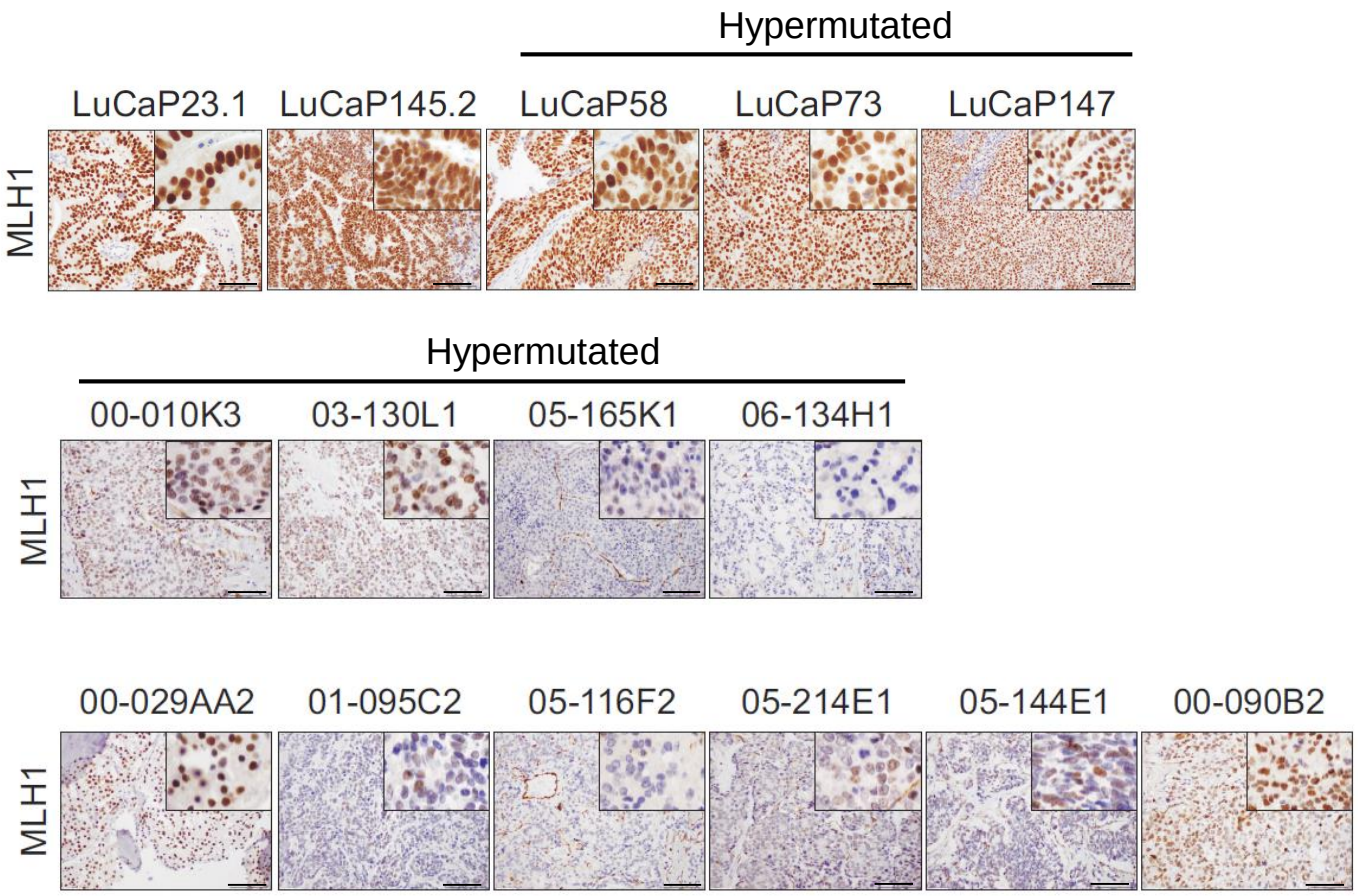
Supplementary Figure 11: IHC results for prostate rapid autopsy metastasis samples. Hypermutated MSI positive autopsy cases 00-010, 03-130, 05-165, which harbored somatic mutations in *MSH2*, *MSH6* or both genes show complete loss of MSH2 and MSH6 expression by IHC using a tissue microarray (top panels). Tissue was not available for IHC studies in hypermutated case 05-123. Hypermutated MSI positive case 06-134, which had somatic deletion of *MLH1*, has focal intact nuclear expression of MSH2 and MSH6 protein (top left, insets). By contrast, MSH2 and MSH6 nuclear staining is intact in MSI-negative autopsy cases 00-029, 01-095, 05-214, and 05-144 (bottom panels, examples of positive nuclear staining in insets). MSH6, but not MSH2 protein expression was detected MSI-negative case 05-116, a case that also had absent MLH1 protein (see separate figure). This could reflect a false negative result due to poor quality tissue for this sample on the tissue microarray. For MSH2 and MSH6, heterogeneity of immunostaining is common in tumor tissue, and protein expression is generally considered intact if any cells display positive nuclear staining. Because MSH2 and MSH6 function as a heterodimer, mutations in one gene frequently result in loss of expression of both proteins, particularly when there are *MSH2* mutations. All samples are from metastases and not primary tumors. Scale bar: 0.1mm.

Supplementary Figure 12

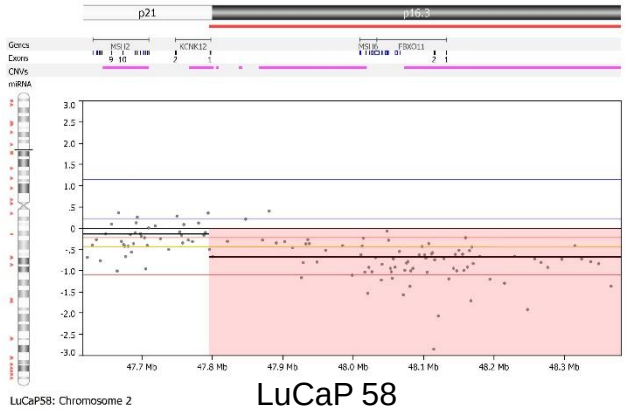


Sample	*MLH1* methylation status
LuCaP 58	Unmethylated
LuCaP 73	Unmethylated
LuCaP 147	Unmethylated
05-165-K3	Unmethylated
03-130-L2	Unmethylated
06-134-P1	Unmethylated
00-010	Unmethylated
05-123-D1	Unmethylated
POS CRC 1 (known methylated colon tumor)	Methylated
POS CRC 2 (known methylated colon tumor)	Methylated
RKO (positive control cell line)	Methylated
SW 480 (negative control cell line)	Unmethylated

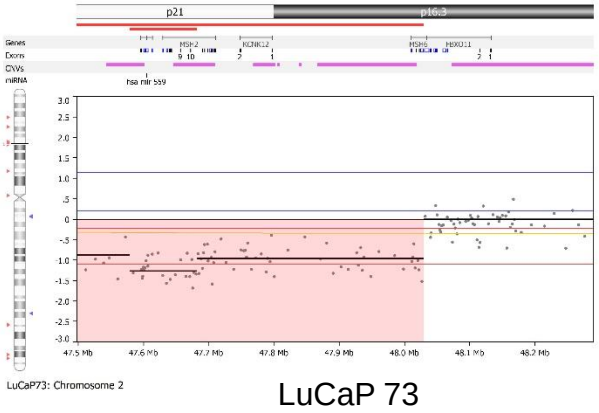
Supplementary Figure 12: Hypermutated Prostate Tumors Do Not Exhibit *MLH1* Methylation. Results of a *MLH1* methylation-specific SYBR green assay are expressed as ratios between Methyl-MLH1 or Unmethyl-MLH1 values and the ALUC4 control values. Genomic DNA samples were bisulfite-treated with EZ DNA Methylation Kit (Zymo Research, Irvine, CA) according to manufacturer's protocol. The primers used in the SYBR Green Assay were previously described (see methods). The error bars represent the standard error of the mean. DNA samples from 2 known *MLH1* methylated colon cancer tumors (POS CRC1 and POS CRC2) and cancer cell lines RKO (methylated) and SW480 (unmethylated) are used as controls for the assay.



Supplementary Figure 13: Hypermutated MSI Positive Prostate Tumors With *MSH2* or *MSH6* mutations have intact MLH1 protein by IHC. Hypermutated MSI positive cases LuCaP 58, LuCaP 73, LuCaP 147 (top) and autopsy cases 00-010, 03-130, 05-165 (middle), which harbored somatic mutations in *MSH2*, *MSH6* or both have intact MLH1 expression by IHC using a tissue microarray. This corroborates the *MLH1* methylation studies and strongly argues against *MLH1* epigenetic silencing as a mechanism of MSI in these tumors. Hypermutated MSI positive case 06-134 that had homozygous deletion of *MLH1* has absent MLH1 protein. Tissue was not available for IHC studies in hypermutated case 05-123. MLH1 protein expression was not detected MSI-negative case 05-116 (bottom), a case that also had absent MSH2 protein (see separate figure). This could reflect a false negative result due to poor quality tissue for this sample on the tissue microarray. For MLH1, heterogeneity of immunostaining is common in tumor tissue, and protein expression is generally considered intact if any cells display positive nuclear staining. Scale bar: 0.1mm.

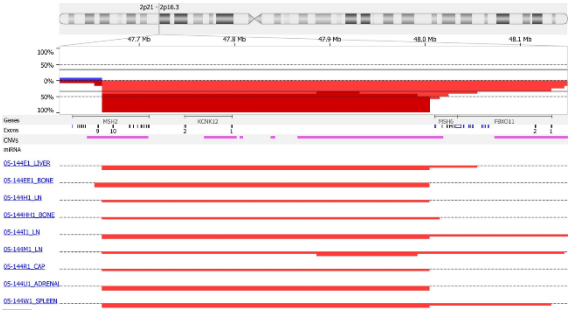


LuCaP 58

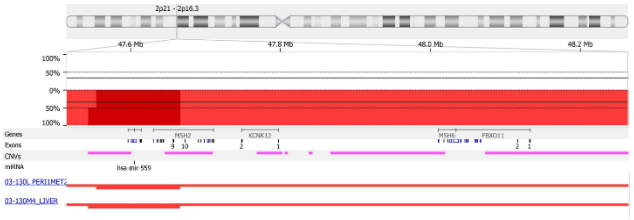


LuCaP 73

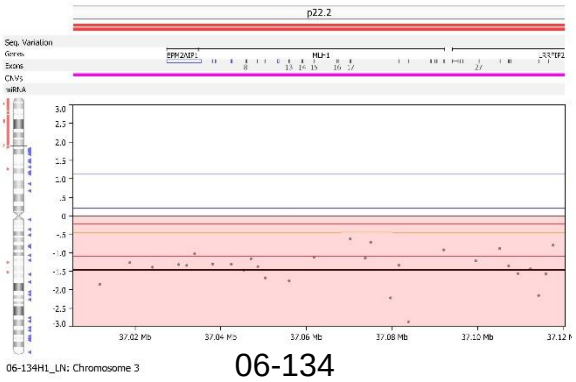
LuCaP 147 and 05-165



05-144



03-130



06-134

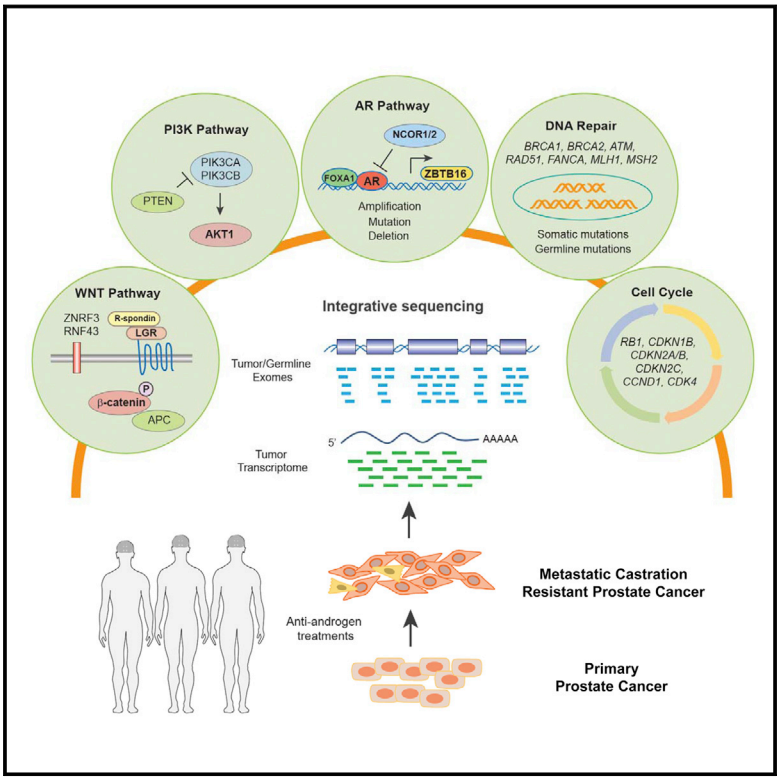
Supplementary Figure 14: Confirmation of *MSH2*, *MSH6*, and *MLH1* Copy Number Status by Genomic Microarray. Genomic microarray (array CGH) was performed for all cases that had *MSH2* and *MSH6* structural rearrangements and also for case 06-134 with *MLH1* homozygous gene deletion. The genomic loci are given along the top of each panel. Y axes are log2 ratio compared to normal control. Red indicates deletion. The results are concordant with copy number status as assessed by BROCA next-generation sequencing.

Resource

Cell

Integrative Clinical Genomics of Advanced Prostate Cancer

Graphical Abstract



Highlights

- A multi-institutional integrative clinical sequencing of mCRPC
- Approximately 90% of mCRPC harbor clinically actionable molecular alterations
- mCRPC harbors genomic alterations in *PIK3CA/B*, *RSPO*, *RAF*, *APC*, β-catenin, and *ZBTB16*
- 23% of mCRPC harbor DNA repair pathway aberrations, and 8% harbor germline findings

Authors

Dan Robinson, Eliezer M. Van Allen, ..., Charles L. Sawyers, Arul M. Chinnaiyan

Correspondence

sawyersc@mskcc.org (C.L.S.), arul@umich.edu (A.M.C.)

In Brief

A multi-institutional integrative clinical sequencing analysis reveals that the majority of affected individuals with metastatic castration-resistant prostate cancer harbor clinically actionable molecular alterations, highlighting the need for genetic counseling to inform precision medicine in affected individuals with advanced prostate cancer.

Robinson et al., 2015, Cell *161*, 1215–1228
May 21, 2015 ©2015 Elsevier Inc.
http://dx.doi.org/10.1016/j.cell.2015.05.001

CellPress

Cell

Integrative Clinical Genomics of Advanced Prostate Cancer

Dan Robinson,[1,2,43] Eliezer M. Van Allen,[3,4,43] Yi-Mi Wu,[1,2] Nikolaus Schultz,[5,40] Robert J. Lonigro,[1] Juan-Miguel Mosquera,[6,7,8,38] Bruce Montgomery,[9,10] Mary-Ellen Taplin,[3] Colin C. Pritchard,[26] Gerhardt Attard,[11,12] Himisha Beltran,[7,8,13,38] Wassim Abida,[14,20] Robert K. Bradley,[9] Jake Vinson,[15] Xuhong Cao,[1,42] Pankaj Vats,[1] Lakshmi P. Kunju,[1,2,17] Maha Hussain,[16,17,18] Felix Y. Feng,[1,17,19] Scott A. Tomlins,[1,2,17,18] Kathleen A. Cooney,[16,17,18] David C. Smith,[16,17,18] Christine Brennan,[1] Javed Siddiqui,[1] Rohit Mehra,[1,2] Yu Chen,[13,14,20] Dana E. Rathkopf,[13,20] Michael J. Morris,[13,20] Stephen B. Solomon,[21] Jeremy C. Durack,[21] Victor E. Reuter,[22] Anuradha Gopalan,[22] Jianjiong Gao,[40] Massimo Loda,[3,4,23,39] Rosina T. Lis,[3,23] Michaela Bowden,[3,23,39] Stephen P. Balk,[24] Glenn Gaviola,[25] Carrie Sougnez,[4] Manaswi Gupta,[4] Evan Y. Yu,[10] Elahe A. Mostaghel,[9,10] Heather H. Cheng,[9,10] Hyojeong Mulcahy,[27] Lawrence D. True,[28] Stephen R. Plymate,[10] Heidi Dvinge,[9] Roberta Ferraldeschi,[11,12] Penny Flohr,[11,12] Susana Miranda,[11,12] Zafeiris Zafeiriou,[11,12] Nina Tunariu,[11,12] Joaquin Mateo,[11,12] Raquel Perez-Lopez,[11,12] Francesca Demichelis,[7,29] Brian D. Robinson,[6,7,8,38] Marc Schiffman,[7,31,38] David M. Nanus,[7,8,13,38] Scott T. Tagawa,[7,8,13,38] Alexandros Sigaras,[7,30,32] Kenneth W. Eng,[7,30,32] Olivier Elemento,[30] Andrea Sboner,[6,7,30,38] Elisabeth I. Heath,[33,34] Howard I. Scher,[13,20] Kenneth J. Pienta,[35] Philip Kantoff,[3,44] Johann S. de Bono,[11,12,44] Mark A. Rubin,[6,7,8,38,44] Peter S. Nelson,[10,36,37,38,44] Levi A. Garraway,[3,4,44] Charles L. Sawyers,[14,41,44,*] and Arul M. Chinnaiyan[1,2,17,18,42,44,*]

[1]Michigan Center for Translational Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA
[2]Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA
[3]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA
[4]Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA 02142, USA
[5]Department of Epidemiology and Biostatistics, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
[6]Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, New York, NY 10021, USA
[7]Institute for Precision Medicine, Weill Medical College of Cornell University, New York, NY 10021, USA
[8]New York Presbyterian Hospital, New York, NY 10021, USA
[9]Computational Biology Program, Public Health Sciences Division and Basic Science Division, Fred Hutchinson Cancer Center, University of Washington, Seattle, WA 98109, USA
[10]Department of Medicine and VAPSHCS, University of Washington, Seattle, WA 98109, USA
[11]Cancer Biomarkers Team, Division of Clinical Studies, The Institute of Cancer Research, London SM2 5NG, UK
[12]Prostate Cancer Targeted Therapy Group and Drug Development Unit, The Royal Marsden NHS Foundation Trust, London SM2 5NG, UK
[13]Department of Medicine, Weill Medical College of Cornell University, New York, NY 10021, USA
[14]Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
[15]Prostate Cancer Clinical Trials Consortium, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
[16]Department of Internal Medicine, Division of Hematology Oncology, University of Michigan Medical School, Ann Arbor, MI 48109, USA
[17]Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI 48109, USA
[18]Department of Urology, University of Michigan Medical School, Ann Arbor, MI 48109, USA
[19]Department of Radiation Oncology, University of Michigan Medical School, Ann Arbor, MI 48109, USA
[20]Genitourinary Oncology Service, Department of Medicine, Sidney Kimmel Center for Prostate and Urologic Cancers, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
[21]Interventional Radiology, Department of Radiology Service, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
[22]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
[23]Center for Molecular Oncologic Pathology, Dana-Farber Cancer Institute, Boston, MA 02215, USA
[24]Division of Hematology-Oncology, Department of Medicine, Beth Israel Deaconess Cancer Center, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA
[25]Department of Musculoskeletal Radiology, Brigham and Women's Hospital, Boston, MA 02115, USA
[26]Department of Laboratory Medicine, University of Washington, Seattle, WA 98195, USA
[27]Department of Radiology, University of Washington, Seattle, WA 98109, USA
[28]Department of Pathology, University of Washington Medical Center, Seattle, WA 98109, USA
[29]Laboratory of Computational Oncology, CIBIO, Centre for Integrative Biology, University of Trento, 38123 Mattarello TN, Italy
[30]Institute for Computational Biomedicine, Department of Physiology and Biophysics, Weill Medical College of Cornell University, New York, NY 10021, USA
[31]Division of Interventional Radiology, Department of Radiology, New York-Presbyterian Hospital/Weill Cornell Medical Center, New York, NY 10021, USA
[32]Department of Physiology & Biophysics, Weill Medical College of Cornell University, New York, NY 10021, USA
[33]Department of Oncology, Wayne State University School of Medicine, Detroit, MI 48201, USA
[34]Molecular Therapeutics Program, Barbara Ann Karmanos Cancer Institute, Detroit, MI 48201, USA
[35]The James Buchanan Brady Urological Institute and Department of Urology, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA
[36]Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA
[37]Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA
[38]Meyer Cancer, Weill Medical College of Cornell University, New York, NY 10021, USA

[39]Department of Pathology, Brigham & Women's Hospital, Boston, MA 02115, USA
[40]Marie-Josée and Henry R. Kravis Center for Molecular Oncology, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
[41]Howard Hughes Medical Institute, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
[42]Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109, USA
[43]Co-first author
[44]Co-senior author
*Correspondence: sawyersc@mskcc.org (C.L.S.), arul@umich.edu (A.M.C.)
http://dx.doi.org/10.1016/j.cell.2015.05.001

SUMMARY

Toward development of a precision medicine framework for metastatic, castration-resistant prostate cancer (mCRPC), we established a multi-institutional clinical sequencing infrastructure to conduct prospective whole-exome and transcriptome sequencing of bone or soft tissue tumor biopsies from a cohort of 150 mCRPC affected individuals. Aberrations of *AR*, ETS genes, *TP53*, and *PTEN* were frequent (40%–60% of cases), with *TP53* and *AR* alterations enriched in mCRPC compared to primary prostate cancer. We identified new genomic alterations in *PIK3CA/B*, R-spondin, *BRAF/RAF1*, *APC*, β-catenin, and *ZBTB16/PLZF*. Moreover, aberrations of *BRCA2*, *BRCA1*, and *ATM* were observed at substantially higher frequencies (19.3% overall) compared to those in primary prostate cancers. 89% of affected individuals harbored a clinically actionable aberration, including 62.7% with aberrations in AR, 65% in other cancer-related genes, and 8% with actionable pathogenic germline alterations. This cohort study provides clinically actionable information that could impact treatment decisions for these affected individuals.

INTRODUCTION

Prostate cancer is among the most common adult malignancies, with an estimated 220,000 American men diagnosed yearly (American Cancer Society, 2015). Some men will develop metastatic prostate cancer and receive primary androgen deprivation therapy (ADT). However, nearly all men with metastatic prostate cancer develop resistance to primary ADT, a state known as metastatic castration-resistant prostate cancer (mCRPC). Multiple ''second generation'' ADT treatments, like abiraterone acetate (de Bono et al., 2011; Ryan et al., 2013) and enzalutamide (Beer et al., 2014; Scher et al., 2012), have emerged for mCRPC affected individuals; however, nearly all affected individuals will also develop resistance to these agents. In the U.S., an estimated 30,000 men die of prostate cancer yearly.

Multiple studies have identified recurrent somatic mutations, copy number alterations, and oncogenic structural DNA rearrangements (chromoplexy) in primary prostate cancer (Baca et al., 2013; Barbieri et al., 2012; Berger et al., 2011; Cooper et al., 2015; Pflueger et al., 2011; Taylor et al., 2010; Tomlins et al., 2007; Wang et al., 2011). These include point mutations in *SPOP*, *FOXA1*, and *TP53*; copy number alterations involving *MYC*, *RB1*, *PTEN*, and *CHD1*; and E26 transformation-specific (ETS) fusions, among other biologically relevant genes. Although certain primary prostate cancer alterations or signatures have prognostic clinical significance (Hieronymus et al., 2014; Lalonde et al., 2014), the therapeutic impact of primary prostate cancer genomic events has not yet been realized.

Genomic studies of metastatic prostate cancers demonstrated additional alterations in *AR* (Taplin et al., 1995) and in the androgen signaling pathway (Beltran et al., 2013; Grasso et al., 2012; Gundem et al., 2015; Hong et al., 2015), although these studies were performed predominantly using autopsy samples or preclinical models with limited cohort sizes. Prospective genomic characterization of fresh biopsy samples from living mCRPC affected individuals has been limited due to challenges in obtaining adequate tumor tissue, especially from bone biopsies (Mehra et al., 2011; Van Allen et al., 2014a), which is the most common site of metastatic disease. Thus, the landscape of genomic alterations in mCRPC disease remains incompletely characterized. Moreover, the low frequency of actionable genomic alterations in primary prostate cancer has limited the inclusion of mCRPC among cohorts wherein precision cancer medicine approaches have been piloted to guide treatment or clinical trial enrollment.

We conducted a systematic and multi-institutional study of mCRPC tumors obtained from living affected individuals to determine the landscape of somatic genomic alterations in this cohort, dissect genomic differences between primary prostate cancer and mCRPC, and discover the potential relevance of these findings from a biological and clinical perspective.

RESULTS

Clinical, Biopsy, and Pathology Parameters

An international consortium consisting of eight academic medical center clinical sites was established to capture fresh clinical mCRPC affected individual samples as part of standard-of-care approaches or through a cohort of prospective clinical trials (Figures 1A and 1B). Standard-of-care approaches for mCRPC included abiraterone acetate or enzalutamide. Clinical trials included in this study focused on combination strategies involving abiraterone acetate or enzalutamide, inhibitors of poly ADP ribose polymerase (PARP), or inhibitors of aurora kinase. Here, we report the results of genomic profiling from

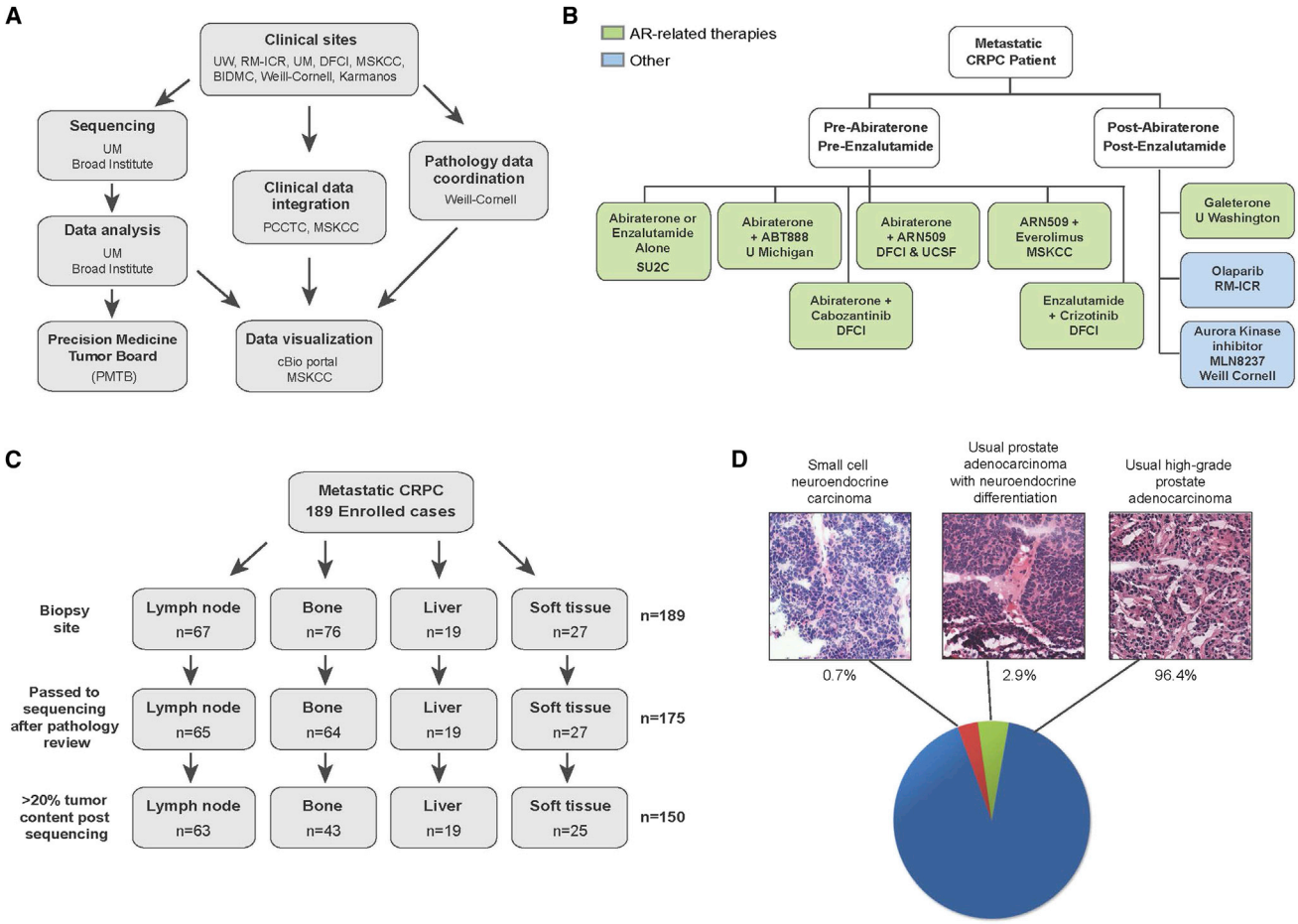


Figure 1. Overview of the SU2C-PCF IDT Multi-Institutional Clinical Sequencing of the mCRPC Project

(A) Schema of multi-institutional clinical sequencing project work flow.

(B) Clinical trials associated with the SU2C-PCF mCRPC project.

(C) Biopsy sites of the samples used for clinical sequencing.

(D) Histopathology of the cohort. Representative images of morphological analysis of mCRPC are shown along with prevalence in our cohort.

mCRPC biopsy samples obtained at time of entry into the cohort study. Future reports will include longitudinal clinical data such as treatment response. The consortium utilized two sequencing and analysis centers, one centralized digital pathology review center, and one centralized data visualization portal (Cerami et al., 2012; Gao et al., 2013; Robinson et al., 2011; Thorvaldsdóttir et al., 2013). Cross-validation of sequencing data from the two original sequencing sites demonstrated comparable variant calls for adequately powered genetic loci (E.M.V.A., D.R., C. Morrissey, C.C.P., S.L. Carter, M. Rosenberg, A. McKenna, A.M.C., L.A.G., and P.S.N., unpublished data).

Here, we describe 150 affected individuals with metastatic disease with complete integrative clinical sequencing results (whole-exome, matched germline, and transcriptome data) (Figure 1C) and summarized in Table S1. 189 affected individuals were enrolled in the study, and 175 cases were sequenced after pathology review and assessment of tumor content. Of these, 150 biopsies had >20% tumor content as defined by computational analysis, based on mutant allele variant fractions and zygosity shifts. The biopsies sequenced were from lymph node (42%), bone (28.7%), liver (12.7%), and other soft tissues (16.7%). Baseline clinical information is available in Table S2. A majority of cases (96.4%) displayed typical high-grade prostate adenocarcinoma features, whereas 2.9% of cases showed neuroendocrine differentiation. One case (0.7%) exhibited small-cell neuroendocrine features (Epstein et al., 2014) (Figure 1D).

Landscape of mCRPC Alterations

Somatic aberrations in a panel of 38 statistically or clinically significant genes are illustrated in Figure 2. Mean target coverage for tumor exomes was 160× and for matched normal exomes was 100×. Although the average mutation rate for mCRPC was 4.4 mutations/Mb, there were four cases that exhibited a mutation rate of nearly 50 per Mb, three of which are likely due to alterations in the mismatch repair genes *MLH1* and *MSH2*, as discussed later.

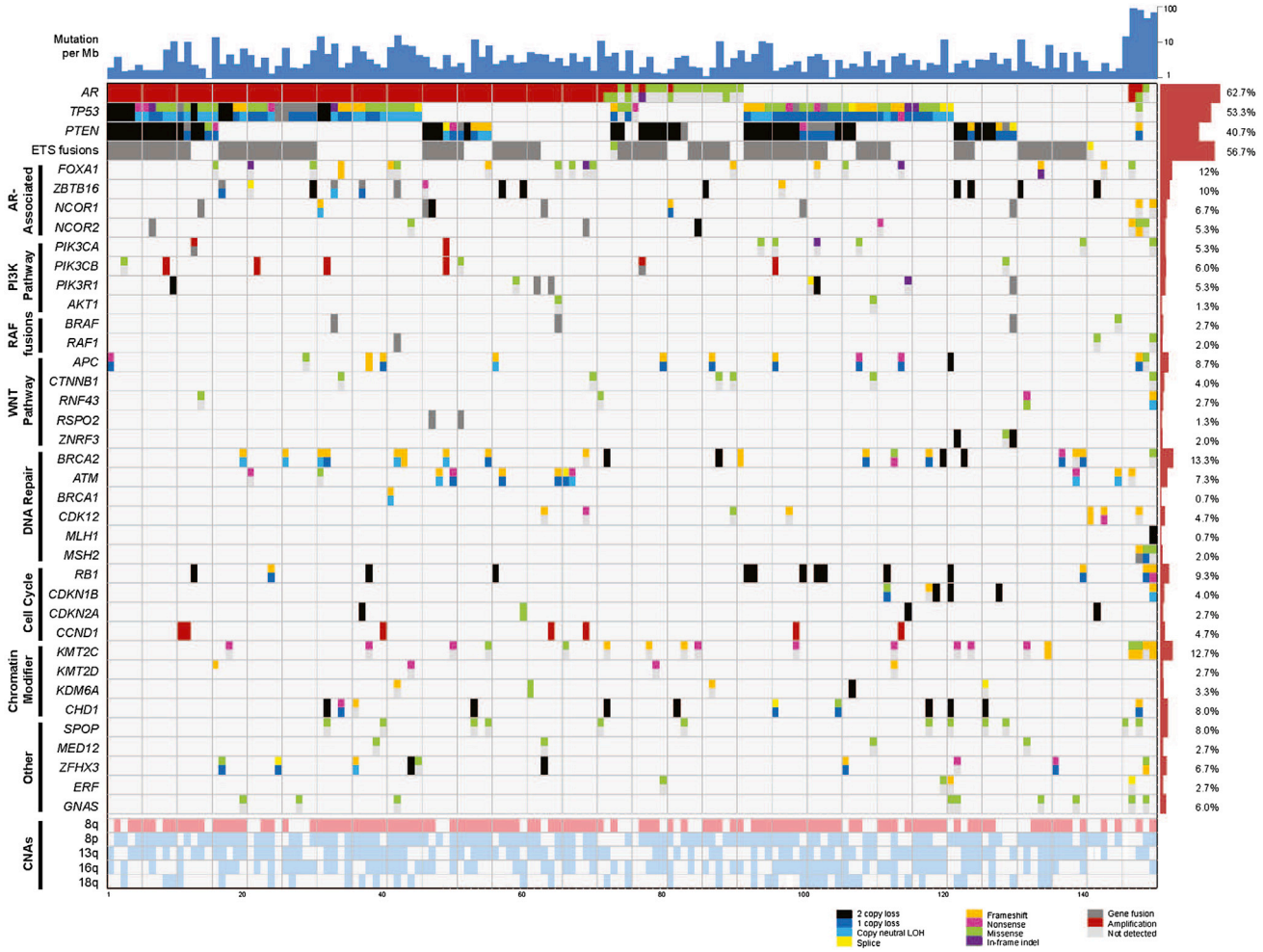


Figure 2. Integrative Landscape Analysis of Somatic and Germline Aberrations in Metastatic CRPC Obtained through DNA and RNA Sequencing of Clinically Obtained Biopsies

Columns represent individual affected individuals, and rows represent specific genes grouped in pathways. Mutations per Mb are shown in the upper histogram, and incidence of aberrations in the cohort is in the right histogram. Copy number variations (CNVs) common to mCRPC are shown in in the lower matrix, with pink representing gain and light blue representing loss. Color legend of the aberrations represented including amplification, two copy loss, one copy loss, copy neutral loss of heterozygosity (LOH), splice site mutation, frameshift mutation, missense mutation, in-frame indel, and gene fusion. Cases with more aberration in a gene are represented by split colors.

Frequent copy number gains of 8q, as well as copy number losses of 8p, 13q, 16q, and 18q, were also observed. The mean number of identified biologically relevant genetic aberrations per case was 7.8 (Figure 2). All mutations identified are presented in Table S3. The landscape of copy number alterations demonstrated expected recurrent amplification peaks (frequent *AR*, 8q gain) and deletion peaks (*CHD1*, *PTEN*, *RB1*, *TP53*) (Figure 3A). Additional frequent focal amplifications were observed in regions encompassing *CCND1* and *PIK3CA* and *PIK3CB*. A new recurrent focal homozygous deletion event was observed in chr11q23, encompassing the transcriptional repressor *ZBTB16*.

To identify gene fusions, analysis of 215 transcriptome libraries derived from the 150 tumor RNAs was performed and identified 4,122 chimeras with at least 4 reads spanning the fusion junction. These fusion junctions resulted from 2,247 gene pairs, an average of 15 gene fusions per tumor (Table S4). Among chimeric fusion transcripts identified, recurrent ETS fusions (Tomlins et al., 2005) were observed in 84 cases (56%), of which the majority were fused to *ERG* and others to *FLI1*, *ETV4*, and *ETV5* (Figure 3B). In addition, potential clinically actionable fusions (involving *BRAF*, *RAF1*, *PIK3CA/B*, or *RSPO2*) were seen in eight cases (Figure S1 and covered subsequently).

To place the mCRPC mutation landscape in the context of primary prostate cancer somatic genomics, we performed a selective enrichment analysis to compare somatic point mutations and short insertion/deletions observed in this cohort with those observed in somatic whole-exome mutation data from 440 primary prostate cancer exomes (Barbieri et al., 2012; The Cancer

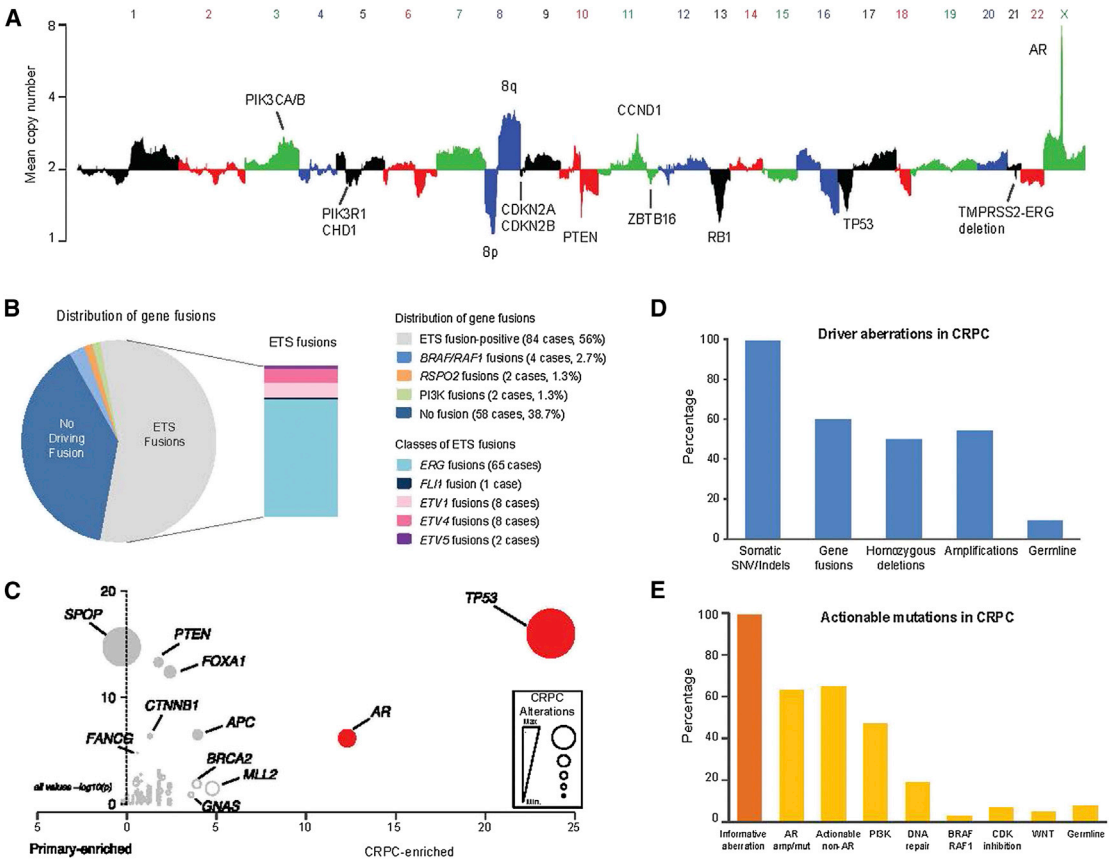


Figure 3. Classes of Genomic Aberrations Seen in mCPRC

(A) Copy number landscape of the SU2C-PCF mCRPC cohort. Individual chromosomes are represented by alternating colors, and key aberrant genes are indicated.

(B) The gene fusion landscape of mCRPC. Pie chart of all driver fusions identified and the box plot represents specific ETS fusions.

(C) Mutations enriched in mCRPC relative to hormone naive primary prostate cancer. Primary prostate cancer data derived from published studies (Barbieri et al., 2012; The Cancer Genome Atlas, 2015). Level of CRPC enrichment is represented by the x axis, and MutSig CRPC significance analysis is provided by the y axis. Diameters are proportional to the number of cases with the specific aberration. Genes of interest are highlighted.

(D) Classes of driver aberrations identified in mCRPC.

(E) Classes of clinically actionable mutations identified in mCRPC.

Genome Atlas, 2015) (Figure 3C and Table S5). Focusing on genes previously implicated in cancer (n = 550), somatic *TP53* mutations were the most selectively mutated ($q < 0.001$; Benjamini-Hochberg), followed by *AR*, *KMT2D*, *APC*, *BRCA2*, and *GNAS* ($q < 0.1$; Benjamini-Hochberg; Table S6). Both *AR* and *GNAS* were mutated exclusively in mCRPC. We found no genes selectively mutated in primary prostate cancer compared to mCRPC.

We identified an established biological "driver" aberration in a cancer-related gene (i.e., known oncogene or tumor suppressor; Table S7) in nearly all the cases (Figure 3D). Although 99% of the mCPRC cases harbored a potential driver single-nucleotide variant (SNV) or indel, other classes of driver aberrations were also highly prevalent. These include driver gene fusions in 60%, driver homozygous deletions in 50% and driver amplifications in 54%. Although informative mutations were present in virtually all mCRPC cases, 63% harbored aberrations in *AR*, an expected finding in castrate-resistant disease but with higher frequency than in prior reports (Figure 3E). Interestingly, even when *AR* was not considered, 65% of cases harbored a putatively clinically actionable alteration (defined as predicting response or resistance to a therapy, having diagnostic or prognostic utility across tumor types) (Table S8) (Roychowdhury et al., 2011; Van Allen et al., 2014c). Non-AR related clinically actionable alterations included aberrations in the PI3K pathway (49%), DNA repair pathway (19%), RAF kinases (3%), CDK inhibitors (7%), and the WNT pathway (5%). In addition to somatic alterations, clinically actionable pathogenic germline variants were seen in 8% of mCRPC affected individuals, potentially emphasizing the need for genetic counseling in affected individuals with prostate cancer.

Genomically Aberrant Pathways in mCRPC

Integrative analysis using both biological and statistical frameworks (Lawrence et al., 2013, 2014) of somatic point mutations,

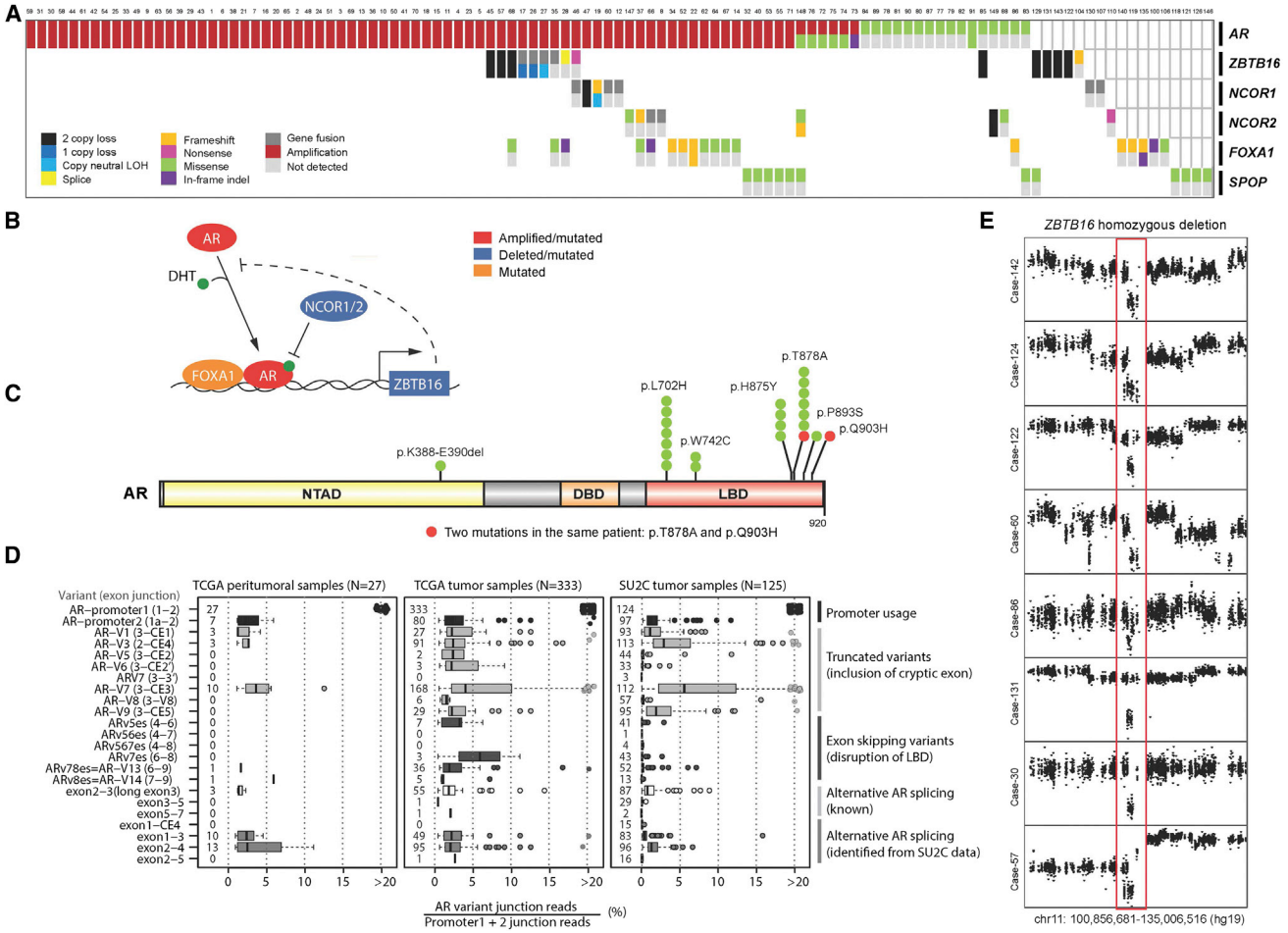


Figure 4. Aberrations in the AR Pathway Found in mCRPC

(A) Cases with aberrations in the AR pathway. Case numbering as in Figure 2.

(B) Key genes found altered in the AR pathway of mCRPC. DHT, dihydrotestosterone.

(C) Point mutations identified in *AR*. Amino acids altered are indicated. NTAD, N-terminal activation. DBD, DNA-binding. LBD, ligand binding.

(D) Splicing landscape of *AR* in mCRPC. Specific splice variants are indicated by exon boundaries, and junction read level is provided. SU2C, this mCRPC cohort. PRAD tumor, primary prostate cancer from the TCGA. PRAD normal, benign prostate from the TCGA.

(E) Homozygous deletion of *ZBTB16*. Copy number plots with x axis representing chromosomal location and the y axis referring to copy number level. Red outline indicates region of *ZBTB16* homozygous loss.

short insertion/deletions, copy number alterations, fusion transcripts, and focused germline variant analysis identified discrete molecular subtypes of mCRPC (Figure 2). These subtypes were classified based on alteration clustering and existing biological pathway knowledge and implicated the *AR* signaling pathway, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), WNT, DNA repair, cell cycle, and chromatin modifier gene sets, among others. The most frequently aberrant genes in mCRPC included *AR* (62.7%), ETS family (56.7%), *TP53* (53.3%), and *PTEN* (40.7%) (Figure 2).

AR Signaling Pathway

In aggregate, 107/150 (71.3%) of cases harbored *AR* pathway aberrations, the majority of which were direct alterations affecting *AR* through amplification and mutation (Figure 4A). Figure 4B summarizes the key genes altered in AR signaling, including *AR* itself, *FOXA1* as a pioneer transcription factor, *NCOR1/2* as negative regulators of AR, *SPOP* as a putative androgen receptor transcriptional regulator (Geng et al., 2013), and *ZBTB16* as an AR inducible target gene that may also negatively regulate *AR*. Recurrent hotspot mutations in *AR* were observed at residues previously reported to confer agonism to AR antagonists such as flutamide (T878A) and bicalutamide (W742C), as well as to glucocorticoids (L702H). Some, but not all, of these affected individuals had documented prior exposures that could explain enrichment for these mutations. Additional clinical data collection is ongoing (Figure 4C). Rare *AR* mutations not previously described were seen in our cohort, although these are of unclear functional significance. Furthermore, one affected individual (Case 89) harbored two putatively

functional *AR* mutations (T878A and Q903H), which may further suggest intra-tumor heterogeneity emerging in the CRPC setting (Carreira et al., 2014). Analysis of *AR* splice variants from RNA-seq data demonstrated a distribution of splice variants observed throughout these mCRPC tumor cases (Figure 4D). Analysis of the TCGA prostate dataset revealed that many of these variants were also present at varying levels in primary prostate cancer and benign prostate tissue. AR-V7, which has been implicated in abiraterone acetate and enzalutamide resistance (Antonarakis et al., 2014), was observed in a majority of pre-abiraterone/enzalutamide cases but at very low ratios relative to full length AR. Implications for treatment response are unknown at this time.

In addition to AR mutations itself, we observed alterations in AR pathway members (Figure 4A). These included known alterations in *NCOR1*, *NCOR2*, and *FOXA1* that have been previously reported in primary prostate cancers and mCRPC (Barbieri et al., 2012; Grasso et al., 2012). In this cohort, truncating and missense mutations in FOXA1 form a cluster near the end of the Forkhead DNA binding domain (Figure S2).

Recurrent homozygous deletions of the androgen-regulated gene *ZBTB16* (also known as PLZF) were seen in 8 (5%) cases (Figure 4E) not previously reported in clinical mCRPC biopsies. Analysis of the minimally deleted region seen in this cohort narrowed the candidate genes in the chr11q23 region to *ZBTB16* (Figure S3). *ZBTB16* has been previously implicated in prostate cancer tumorigenesis and androgen resistance in preclinical models (Cao et al., 2013; Kikugawa et al., 2006), with loss of *ZBTB16* upregulating the MAPK signaling pathway (Hsieh et al., 2015).

New PI3K Pathway Discoveries

The PI3K pathway was also commonly altered, with somatic alterations in 73/150 (49%) of mCRPC affected individuals (Figure 5A). This included biallelic loss of *PTEN*, as well as hotspot mutations, amplifications and activating fusions in *PIK3CA*, and p.E17K activating mutations in *AKT1* (Figure S2). Of note, *PIK3CA* amplifications resulted in overexpression compared to the remaining cohort (Figure S3).

Interestingly, mutations in another member of the PI3K catalytic subunit, *PIK3CB*, were observed in this cohort for the first time, at equivalent positions to canonical activating mutations in PIK3CA (Figure 5B). PIK3CB mutations appeared in the context of PTEN-deficient cases, which is consistent with a previous report demonstrating that some PTEN-deficient cancers are dependent on PIK3CB, rather than PIK3CA (Wee et al., 2008). Furthermore, two affected individuals harbored fusions involving PIK3CA/B, with these events resulting in overexpression of the gene relative to other tumors in the cohort (Figures 5C and 5D).

New Wnt Pathway Discoveries

27/150 (18%) of our cases harbored alterations in the Wnt signaling pathway (Figure 6A). Hotspot activating mutations in *CTNNB1* were seen (Figure 6B), as previously described (Voeller et al., 1998). Notably, recurrent alterations in *APC* were also observed, which have not been previously described in clinical mCRPC affected individuals. This prompted a broader examination of Wnt signaling genes (Figure 6B). Through integrative analysis, we identified alterations in *RNF43* and *ZNRF3*, which were recently described in colorectal, endometrial, and adrenocortical cancers (Assié et al., 2014; Giannakis et al., 2014) and were mutually exclusive with *APC* alterations (Figure 6A). Moreover, we also discovered R-spondin fusions involving *RSPO2*, as previously observed in colorectal carcinoma (Seshagiri et al., 2012) in association with RSPO2 overexpression in these cases (Figure 6C). RSPO2 is a key factor in prostate cancer organoid methodology (Gao et al., 2014). Affected individuals s with aberrations in *RNF43*, *ZNRF3*, or *RSPO2* (overall 6% of affected individuals) are predicted to respond to porcupine inhibitors (Liu et al., 2013).

Cell-Cycle Pathway

We observed *RB1* loss in 21% of cases (Figure S4). Expanding the scope of cell-cycle genes implicated in mCRPC, we noted focal amplifications involving *CCND1* in 9% of cases, as well as less common (< 5%) events in *CDKN2A/B*, *CDKN1B*, and *CDK4* (Figure S4). Cell-cycle derangement, such as through CCND1 amplification or CDKN2A/B loss, may result in enhanced response to CDK4 inhibitors in other tumor types (Finn et al., 2015), and preclinical mCRPC models predict similar activity in prostate cancer (Comstock et al., 2013).

DNA Repair Pathway

Integrative analysis of both the somatic and pathogenic germline alterations in *BRCA2* identified 19/150 (12.7%) of cases with loss of BRCA2, of which ~90% exhibited biallelic loss (Figure 7A). This was commonly a result of somatic point mutation and loss of heterozygosity, as well as homozygous deletion. One of the clinical trials in our consortium is evaluating poly(-ADP-ribose) polymerase (PARP) inhibition in unselected mCRPC affected individuals. Importantly, multiple affected individuals in this trial who experienced clinical benefit harbored biallelic *BRCA2* loss, providing further evidence of clinical actionability (Mateo et al., 2014). Eight affected individuals (5.3%) harbored pathogenic germline *BRCA2* mutations (Figure 7B) with a subsequent somatic event that resulted in biallelic loss, revealing a surprisingly high frequency relative to primary prostate cancer.

We therefore expanded the focus to other DNA repair/recombination genes and identified alterations in at least 34/150 (22.7%) of cases. These include recurrent biallelic loss of *ATM* (Figure 7B), including multiple cases with germline pathogenic alterations. ATM mutations were also observed in affected individuals who achieved clinical responses to PARP inhibition (Mateo et al., 2014). In addition, we noted events in *BRCA1*, *CDK12*, *FANCA*, *RAD51B*, and *RAD51C*. If aberrations of *BRCA2*, *BRCA1*, and *ATM* all confer enhanced sensitivity to PARP inhibitors, 29/150 (19.3%) of mCRPC affected individuals would be predicted to benefit from this therapy. Interestingly, three out of four mCRPC tumors exhibited hypermutation and harbored alterations in the mismatch repair pathway genes *MLH1* or *MSH2* (Figures 2 and 7C), corroborating a recent report identifying structural alterations in *MSH2* and *MSH6* mismatch repair genes in hypermutated prostate cancers (Pritchard et al., 2014).

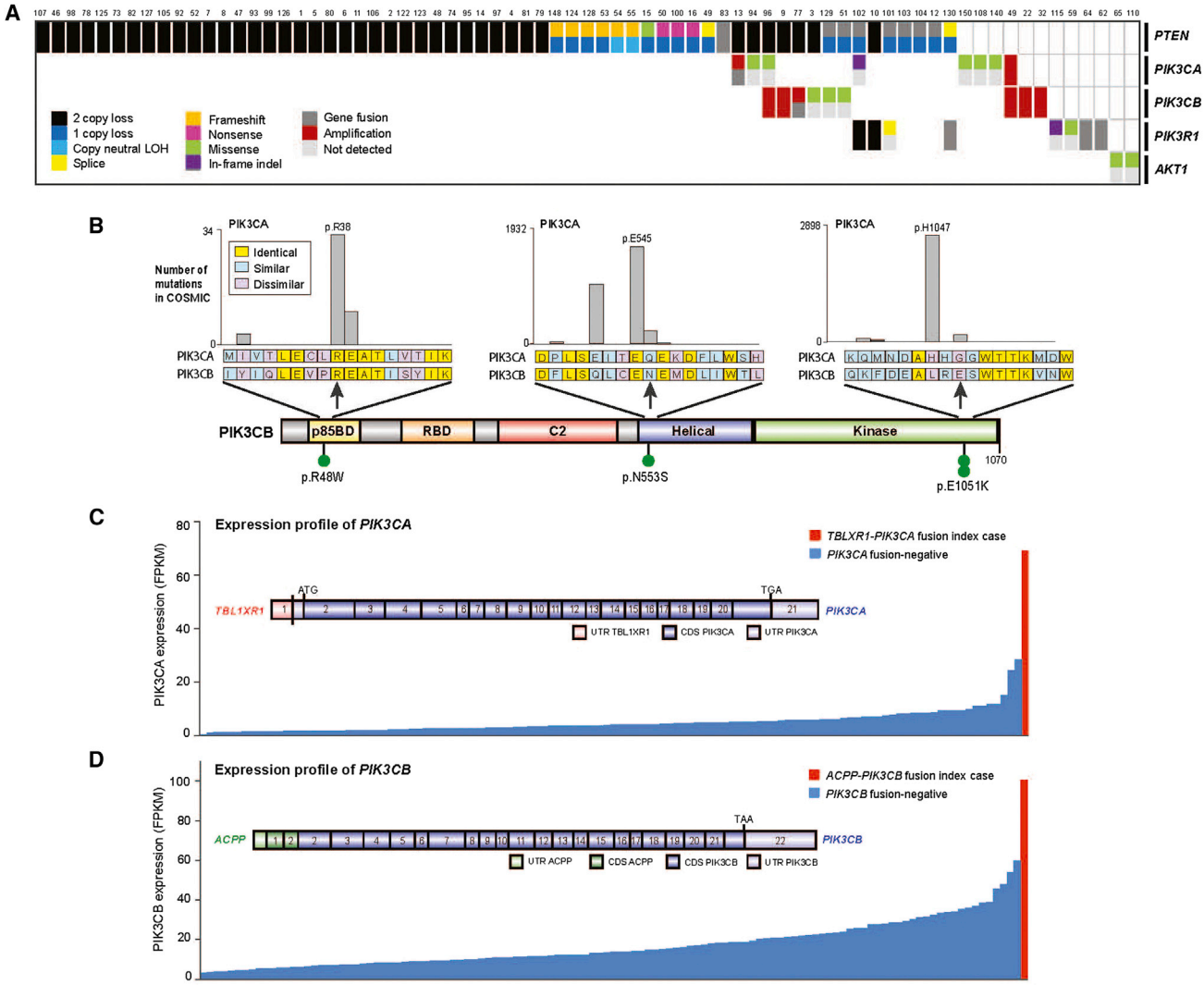


Figure 5. Aberrations in the PI(3)K Pathway Found in mCRPC

(A) Cases with aberrations in the PIK3 pathway. Case numbering as in Figure 2.

(B) Point mutations identified in PIK3CB. Amino acids altered are indicated. Analogous, recurrent COSMIC mutations in PIK3CA are shown as expansion views.

(C) Outlier expression of PK3CA in CRPC case harboring the TBL1XR1-PIK3CA gene fusion. Structure of the gene fusion is inset. UTR, untranslated region. CDS, coding sequence.

(D) As in (C), except for PIK3CB and the ACPP-PIK3CB gene fusion.

DISCUSSION

To effectively implement precision cancer medicine, prospective identification of predictive biomarkers should be performed with information derived from the most contemporary tumor assessments that reflect the affected individual's prior therapies and treatment opportunities. In mCRPC, precision cancer medicine activities have been limited by difficulties obtaining clinical samples from mCRPC affected individuals and a lack of comprehensive genomic data for potentially actionable alterations. By demonstrating the feasibility of prospective genomics in mCRPC and defining the mutational landscape in a focused metastatic clinical cohort, this report may inform multiple genomically driven clinical trials and biological investigations into key mediators of mCRPC. In nearly all of the mCRPC analyzed in this study, we identified biologically informative alterations; almost all harbored at least one driver SNV/indel, and approximately half harbored a driver gene fusion, amplification, or homozygous deletion. Remarkably, in nearly 90% of mCRPC affected individuals, we identified a potentially actionable somatic or germline event.

The high frequency of AR pathway alterations in this cohort strongly implies that the vast majority of mCRPC affected individuals remain dependent on AR signaling for viability. The "second-generation" AR-directed therapies (e.g., abiraterone acetate and enzalutamide) may select for distinct phenotypes that may be indifferent to AR signaling, and prospective

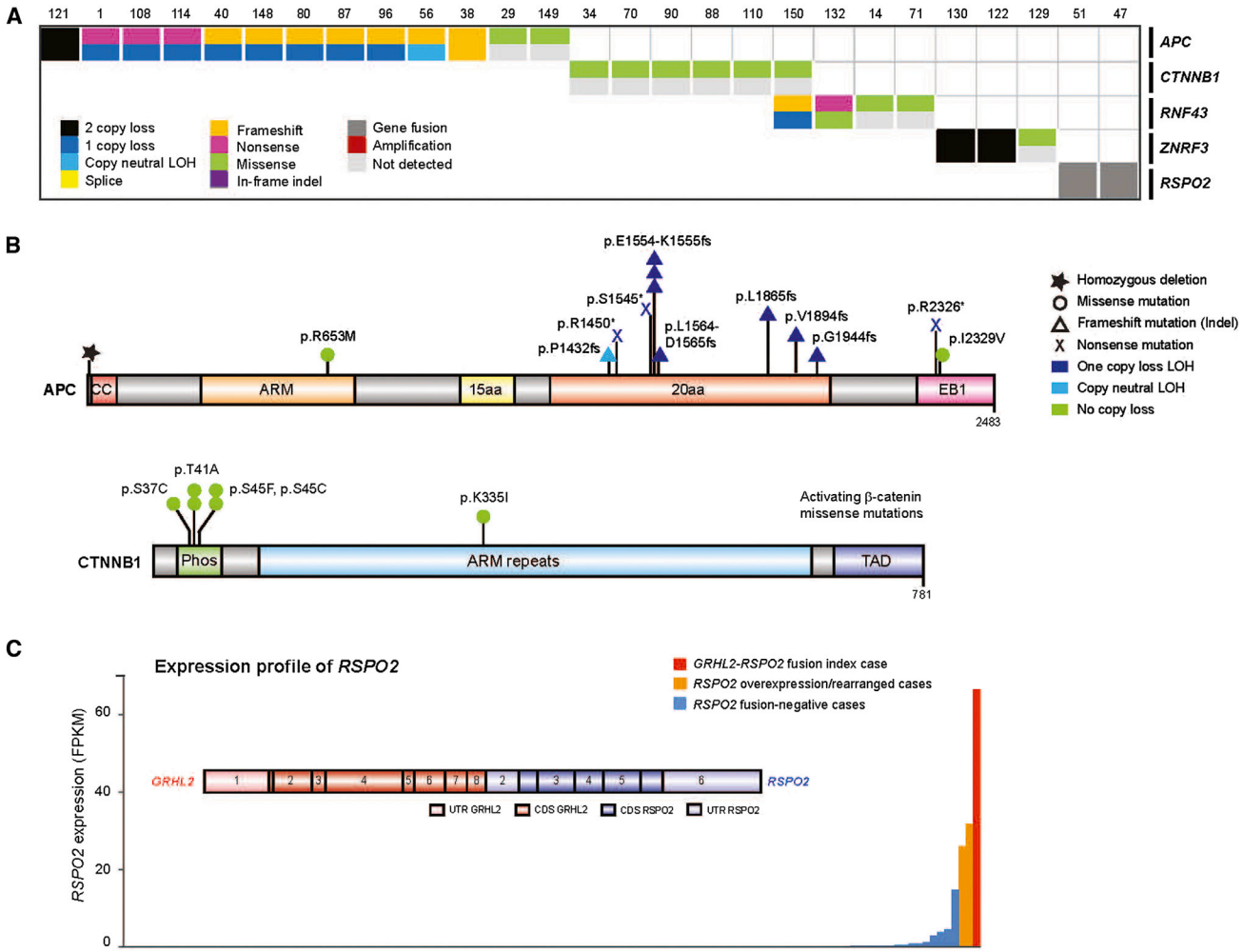


Figure 6. Aberrations in the WNT Pathway Found in mCRPC

(A) Cases with aberrations in the WNT pathway. Case numbering as in Figure 2.

(B) Aberrations identified in APC and CTNNB1. Amino acids altered are indicated. ARM, armadillo repeat. Phos, phosphorylation domain. TAD, *trans*-activating domain. EB1, end binding protein-1 domain. CC, coiled coil.

(C) Outlier expression of *RSPO2* in CRPC and the *GRHL2-RSPO2* gene fusion. RNA-seq expression across our CRPC cohort. Structure of the gene fusion is inset. UTR, untranslated region. CDS, coding sequence.

characterization of such cases will be of particular interest. We hypothesize that affected individuals with acquired *AR* mutations, including new *AR* mutations discovered in this cohort, will harbor differential responses to these second-generation ADT therapies. As the number of affected individuals in this cohort with *AR* mutations increases, we will subsequently be able to link specific AR mutations with clinical phenotypes to determine which mutations confer selective response or resistance to subsequent AR-directed therapy.

Moreover, these data identify multiple therapeutic avenues warranting clinical investigation in the CRPC population. Excluding AR aberrations, 65% of mCRPC have a potentially actionable aberration that may suggest an investigational drug or approved therapy. For example, focusing on the PI3K pathway, PIK3CB-specific inhibitors may have utility in affected individuals with mutation, amplification, and/or fusion of this gene (Schwartz et al., 2015); multiple affected individuals who achieved durable (>1 year) responses to PIK3CB-specificin inhibition harbored activating mutation or amplification in *PIK3CB* (J.S. de Bono et al., 2015, 106th Annual Meeting of the American Association for Cancer Research, abstract). RAF kinase fusions in 3% of mCPRC affected individuals would suggest the use of pan-RAF inhibitors or MEK inhibitors (Palanisamy et al., 2010). In addition, the emergence of porcupine inhibitors (Liu et al., 2013) and R-spondin antibodies may warrant investigation in mCRPC tumors harboring Wnt pathway alterations or specifically R-spondin fusions, respectively. These observations will need to be prospectively assessed in the clinical trials.

Additionally, biallelic inactivation of *BRCA2*, *BRCA1*, or *ATM* was observed in nearly 20% of affected individuals. Previous work in other cancer types suggests that these affected individuals may benefit from PARP inhibitors (Fong et al., 2009;

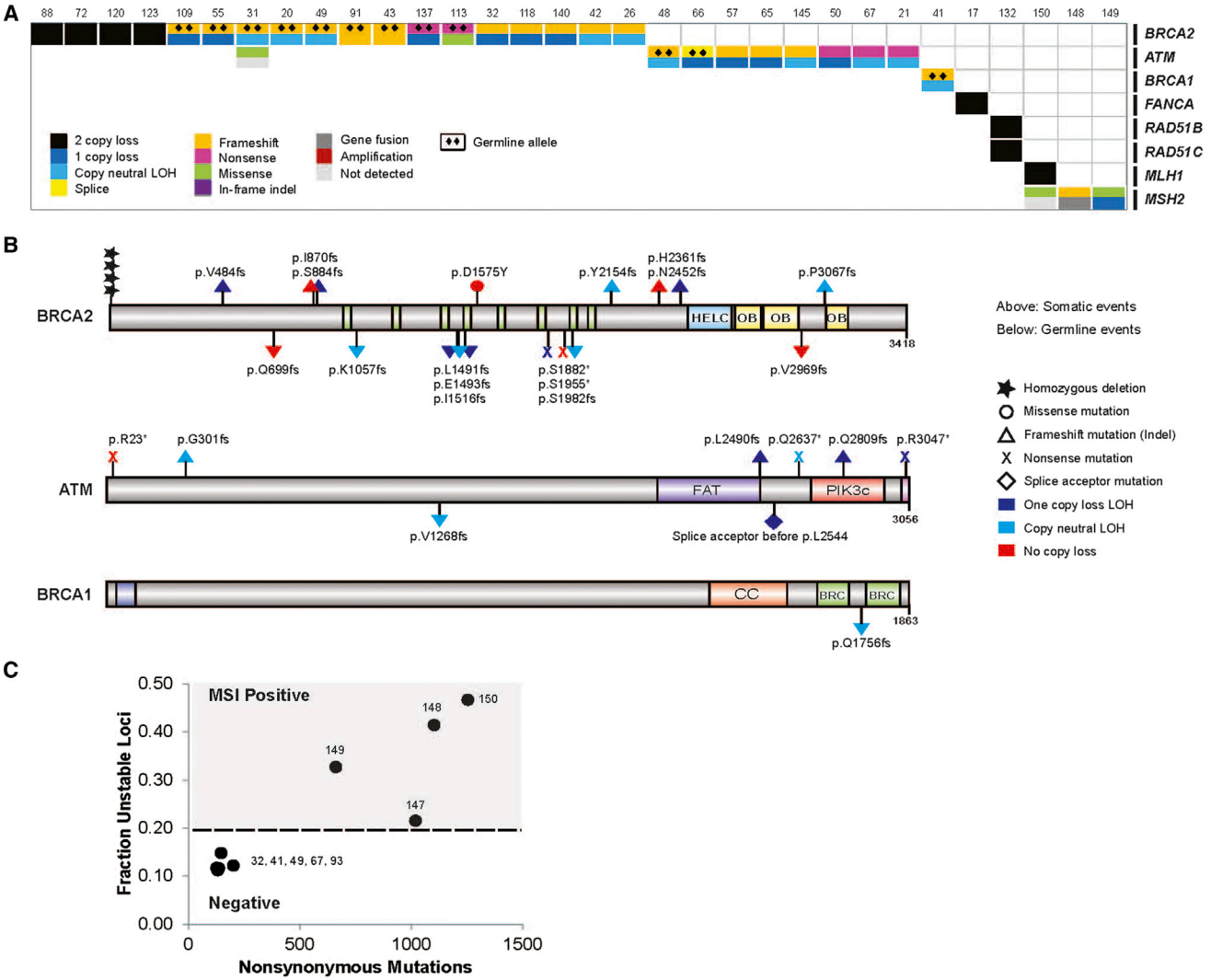


Figure 7. Aberrations in the DNA Repair Pathway Found in mCRPC

(A) Cases with aberrations in the DNA repair pathway. Case numbering as in Figure 2.

(B) Aberrations identified in BRCA2, ATM, and BRCA1. Amino acids altered are indicated. HELC, helical domain. OB, oligonucleotide binding fold. FAT, FRAP-ATM-TRRAP domain. PIK3c, PI3 kinase domain. CC, coiled coil. BRC, Brca repeat.

(C) Microsatellite instability analysis of representative hypermutated CRPC cases and non-hypermutated cases.

Kaufman et al., 2015; Weston et al., 2010) or platinum-based chemotherapy, and prior reports have implicated the presence of germline *BRCA2* alterations in primary prostate cancer with poor survival outcomes (Castro et al., 2013). Given the incidence of pathogenic germline *BRCA2* mutations in this cohort with subsequent somatic events (5%), along with enrichment for somatic *BRCA2* alterations in mCRPC (13%), germline genetic testing in mCRPC affected individuals warrants clinical consideration.

The ability to molecularly characterize mCRPC biopsy samples from affected individuals actively receiving therapy will also enable focused studies of resistance to secondary ADT therapies, including neuroendocrine-like phenotypes. This will require iterative sampling of pre-treatment and resistant tumors from matching affected individuals and may warrant multiregional biopsies from affected individuals (if feasible) given heterogeneity in mCRPC (Carreira et al., 2014; Gundem et al., 2015). Toward that end, in some affected individuals, we observed multiple *AR* mutations emerging in the same biopsy, which may indicate clonal heterogeneity within these mCRPC tumor samples. Additional genomic alterations discovered in this cohort (e.g., *ZBTB16*) warrant exploration in prostate cancer model systems, including organoid cultures (Gao et al., 2014).

Broadly, our effort demonstrates the utility of applying comprehensive genomic principles developed for primary malignancies (e.g., TCGA) to a clinically relevant metastatic tumor cohort. Our effort may also catalyze multi-institutional efforts to profile tumors from cohorts of affected individuals with metastatic, treated tumors in other clinical contexts because our results

demonstrate multiple discoveries within this advanced disease stage that have not been observed in primary tumor profiling. Moreover, this study sets the stage for epigenetic and other profiling efforts in mCRPC not taken in this study, which may enable biological discovery and have immediate therapeutic relevance in mCRPC (Asangani et al., 2014). Overall, our efforts demonstrate the feasibility of comprehensive and integrative genomics on prospective biopsies from individual mCRPC affected individuals to enable precision cancer medicine activities in this large affected individual population.

EXPERIMENTAL PROCEDURES

Affected Individual Enrollment

Affected individuals with clinical evidence of mCRPC who were being considered for abiraterone acetate or enzalutamide as standard of care, or as part of a clinical trial, were considered for enrollment. Affected individuals with metastatic disease accessible by image-guided biopsy were eligible for inclusion. All affected individuals provided written informed consent to obtain fresh tumor biopsies and to perform comprehensive molecular profiling of tumor and germline samples.

Biopsies and Pathology Review

Biopsies of soft tissue or bone metastases were obtained under radiographic guidance. Digital images of biopsy slides were centrally reviewed using schema established to distinguish usual adenocarcinoma from neuroendocrine prostate cancer (Epstein et al., 2014). All images were reviewed by genitourinary oncology pathologists (M.R., J.M.M., L.P.K., S.A.T., R.M., V.R., A.G., M.L., R.L., and M.B.).

Sequencing and Analysis

Normal DNAs from buccal swabs, buffy coats, or whole blood were isolated using the QIAGEN DNeasy Blood & Tissue Kit. Flash-frozen needle biopsies with highest tumor content for each case, as determined by pathology review, were extracted for nucleic acids. Tumor genomic DNA and total RNA were purified from the same sample using the AllPrep DNA/RNA/miRNA kit (QIAGEN) with disruption on a Tissuelyser II (QIAGEN). RNA integrity was verified on an Agilent 2100 Bioanalyzer using RNA Nano reagents (Agilent Technologies).

Whole-exome capture libraries were constructed from 100 ng to 1 μg of DNA from tumor and normal tissue after sample shearing, end repair, and phosphorylation and ligation to barcoded sequencing adaptors. Ligated DNA was size selected for lengths between 200 and 350 bp and subjected to hybrid capture using SureSelect Exome v4 baits (Agilent). Exome sequence data processing and analysis were performed using pipelines at the Broad Institute and the University of Michigan. A BAM file aligned to the hg19 human genome build was produced using Illumina sequencing reads for the tumor and normal sample and the Picard pipeline. Somatic mutation analysis was performed as described previously (Cibulskis et al., 2013; Van Allen et al., 2014c) and reviewed with Integrated Genomics Viewer (IGV) (Robinson et al., 2011).

Copy number aberrations were quantified and reported for each gene as the segmented normalized log2-transformed exon coverage ratios between each tumor sample and matched normal sample (Lonigro et al., 2011). To account for observed associations between coverage ratios and GC content across the genome, lowess normalization was used to correct per-exon coverage ratios prior to segmentation analysis. Mean GC percentage was computed for each targeted region, and a lowess curve was fit to the scatterplot of log2-coverage ratios versus mean GC content across the targeted exome using the lowess function in R (version 2.13.1) with smoothing parameter f = 0.05. The resulting copy ratios were segmented using the circular binary segmentation algorithm (Olshen et al., 2004).

Statistical analysis of recurrently mutated genes was performed using MutSig (Lawrence et al., 2013). Selective enrichment analysis (Van Allen et al., 2014b) of mutations observed in mCRPC compared to primary prostate cancer was performed by tabulating the frequency of affected-individual-normalized mutations observed in either CRPC or primary prostate cancer and performing a two-sided Fisher's exact test using allelic fraction cut off of 0.1 or greater and a set of biologically relevant cancer genes (n = 550 genes) (Futreal et al., 2004). Multiple hypothesis test correction was performed using Benjamini-Hochberg method.

Transcriptome libraries were prepared using 200–1,000 ng of total RNA. PolyA+ RNA isolation, cDNA synthesis, end-repair, A-base addition, and ligation of the Illumina indexed adapters were performed according to the TruSeq RNA protocol (Illumina). Libraries were size selected for 250–300 bp cDNA fragments on a 3% Nusieve 3:1 (Lonza) gel, recovered using QIAEX II reagents (QIAGEN), and PCR amplified using Phusion DNA polymerase (New England Biolabs). Total transcriptome libraries were prepared as above, omitting the poly A selection step and captured using Agilent SureSelect Human All Exon V4 reagents and protocols. Library quality was measured on an Agilent 2100 Bioanalyzer for product size and concentration. Paired-end libraries were sequenced with the Illumina HiSeq 2500, (2×100 nucleotide read length) with sequence coverage to 50 M paired reads and 100 M total reads.

Paired-end transcriptome sequencing reads were aligned to the human reference genome (GRCh37/hg19) using a RNA-seq spliced read mapper Tophat2 (Kim and Salzberg, 2011) (Tophat 2.0.4), with "–fusion-search" option turned on to detect potential gene fusion transcripts. Potential false-positive fusion candidates were filtered out using "Tophat-Post-Fusion" module. Further, the fusion candidates were manually examined for annotation and ligation artifacts. Gene expression, as fragments per kilobase of exon per million fragments mapped (FPKM; normalized measure of gene expression), was calculated using Cufflinks (Trapnell et al., 2012).

ACCESSION NUMBERS

The accession number for the data reported in this paper is dbGap: phs000915.v1.p1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.05.001.

AUTHOR CONTRIBUTIONS

Y-M.W., N.S., R.J.L., J.-M.M., R.M., M.E.T., C.C.P., G.A., H.B., and W.A. made equal contributions. D.R., E.M.V.A., and R.J.L. coordinated overall sequencing and bioinformatics analysis. Y.M.W., D.R., E.M.V.A., R.J.L., W.A., and J.V. coordinated figures and tables. N.S. developed the SU2C-PCF IDT cBio portal. R.J.L. coordinated copy number analyses. J.-M.M. coordinated central pathology review, and L.P.K coordinated UM pathology analysis. C.C.P. coordinated hypermutation analysis and clinical germline interpretations. R.M., M.E.T, G.A., H.B., M.H., H.I.S., and E.I.H. coordinated clinical enrollment at their specific sites. R.K.B., S.P., and H.D. carried out AR splice variant analysis. J.V. managed the clinical data portal. X.C., J.S., P.F., S.M., and C.S. were involved in project management. R.M., S.A.T., V.E.R., A.G., M.L., S.P.B., R.T.L., M.B., B.D.R., J.-M.M, M.R., and L.D.T. were involved in pathology review. C.B., P.V., J.G., M.G., F.D., O.E., A. Sboner, A. Sigaras, and K.W.E. contributed to bioinformatics analysis. K.A.C., D.C.S., F.Y.F., H.I.S., D.R., S.B.S., M.J.M., R.F., Z.Z., N.T., G.G., J.C.D., J.M., D.M.N., S.T.T., E.Y.Y., Y.C., E.A.M., H.H.C., M.A.S., and K.J.P. are clinical contributors. E.M.V.A., D.R., C.L.S. and A.M.C. wrote the manuscript, which all authors reviewed. P.K., J.S.d.B., M.A.R., P.S.N., and L.A.G. are SU2C-PCF Dream Team Principals, and C.L.S. and A.M.C. are Dream Team co-Leaders.

ACKNOWLEDGMENTS

We thank the affected individuals who participated in this study to better understand the feasibility and utility of precision medicine approaches for advanced prostate cancer. Individuals at our respective institutions who helped with this study are listed by institution. University of Michigan: Karen Giles, Lynda Hodges, Erica Rabban, Ning Yu, Fengyun Su, Rui Wang, Brendan Veeneman, and Moshe Talpaz. MSKCC: Brett Carver, Kristen Curtis, and Julie

Filipenko. DFCI/Broad: Zhenwei Zhang, Daniele Depalo, and Joseph Kramkowski. University of Washington: Jina Taub, Hiep Nguyen, Colm Morrissey, and Robert Vessella. ICR/Royal Marsden: Suzanne Carreira, Ines Figueiredo, and Daniel Nava Rodrigues. This work was supported by a Stand Up To Cancer-Prostate Cancer Foundation Prostate Dream Team Translational Cancer Research Grant. Stand Up To Cancer is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research (SU2C-AACR-DT0712). The project was also supported by the following NIH awards: Clinical Sequencing Exploratory Research (CSER) UM1HG006508 (A.M.C.), Early Detection Research Network grant UO1 CA111275(A.M.C.), Prostate SPORE grants P50 CA186786 (A.M.C.), P50 CA092629 (H.I.S., C.L.S., and Y.C.), P50 CA90381 (P.K.),and P50 CA097186 (P.S.N., B.M., E.A.M., and L.D.T.), P01 CA163227 (P.S.N., S.P., R.K.B., H.D.), R01 CA116337 (M.A.R., H.B., F.D.), R01 CA155169 (C.L.S.), R01 CA092629, P50 CA092629 (H.I.S., C.L.S., and Y.C.), and R01 CA155169 (C.L.S.). This work was supported by the following DoD awards: W81XWH-09-1-0147 (PCCTC) and DOD PC121341 (H.B.). This work was also supported by the Starr Cancer Consortium (N.S., M.R., C.S., Y.C., L.A.G.). A.M.C. is an A. Alfred Taubman Scholar and an American Cancer Society Professor. H.B. is supported by Damon Runyon Cancer Research Foundation CI-67-13. C.P. is supported by a PCF Young Investigator Award and DoD PC131820. E.M.V. is supported by a NIH 1K08CA188615. E.M.V., N.S., and F.Y.F. are supported by Prostate Cancer Foundation Young Investigator Awards. The RM and ICR team is supported by the Movember Foundation and Prostate Cancer UK, PCF, the ECMC network from Cancer Research UK, the Department of Health in the UK, and BRC grant funding.

Received: March 9, 2015
Revised: April 6, 2015
Accepted: April 27, 2015
Published: May 21, 2015

REFERENCES

American Cancer Society (2015). Cancer Facts and Figures 2015. http://www.cancer.org/acs/groups/content/@editorial/documents/document/acspc-044552.pdf.

Antonarakis, E.S., Lu, C., Wang, H., Luber, B., Nakazawa, M., Roeser, J.C., Chen, Y., Mohammad, T.A., Chen, Y., Fedor, H.L., et al. (2014). AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N. Engl. J. Med. *371*, 1028–1038.

Asangani, I.A., Dommeti, V.L., Wang, X., Malik, R., Cieslik, M., Yang, R., Escara-Wilke, J., Wilder-Romans, K., Dhanireddy, S., Engelke, C., et al. (2014). Therapeutic targeting of BET bromodomain proteins in castration-resistant prostate cancer. Nature *510*, 278–282.

Assié, G., Letouzé, E., Fassnacht, M., Jouinot, A., Luscap, W., Barreau, O., Omeiri, H., Rodriguez, S., Perlemoine, K., René-Corail, F., et al. (2014). Integrated genomic characterization of adrenocortical carcinoma. Nat. Genet. *46*, 607–612.

Baca, S.C., Prandi, D., Lawrence, M.S., Mosquera, J.M., Romanel, A., Drier, Y., Park, K., Kitabayashi, N., MacDonald, T.Y., Ghandi, M., et al. (2013). Punctuated evolution of prostate cancer genomes. Cell *153*, 666–677.

Barbieri, C.E., Baca, S.C., Lawrence, M.S., Demichelis, F., Blattner, M., Theurillat, J.P., White, T.A., Stojanov, P., Van Allen, E., Stransky, N., et al. (2012). Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. Nat. Genet. *44*, 685–689.

Beer, T.M., Armstrong, A.J., Rathkopf, D.E., Loriot, Y., Sternberg, C.N., Higano, C.S., Iversen, P., Bhattacharya, S., Carles, J., Chowdhury, S., et al.; PREVAIL Investigators (2014). Enzalutamide in metastatic prostate cancer before chemotherapy. N. Engl. J. Med. *371*, 424–433.

Beltran, H., Yelensky, R., Frampton, G.M., Park, K., Downing, S.R., MacDonald, T.Y., Jarosz, M., Lipson, D., Tagawa, S.T., Nanus, D.M., et al. (2013). Targeted next-generation sequencing of advanced prostate cancer identifies potential therapeutic targets and disease heterogeneity. Eur. Urol. *63*, 920–926.

Berger, M.F., Lawrence, M.S., Demichelis, F., Drier, Y., Cibulskis, K., Sivachenko, A.Y., Sboner, A., Esgueva, R., Pflueger, D., Sougnez, C., et al. (2011). The genomic complexity of primary human prostate cancer. Nature *470*, 214–220.

Cao, J., Zhu, S., Zhou, W., Li, J., Liu, C., Xuan, H., Yan, J., Zheng, L., Zhou, L., Yu, J., et al. (2013). PLZF mediates the PTEN/AKT/FOXO3a signaling in suppression of prostate tumorigenesis. PLoS ONE *8*, e77922.

Carreira, S., Romanel, A., Goodall, J., Grist, E., Ferraldeschi, R., Miranda, S., Prandi, D., Lorente, D., Frenel, J.S., Pezaro, C., et al. (2014). Tumor clone dynamics in lethal prostate cancer. Sci. Transl. Med. *6*, 254ra125.

Castro, E., Goh, C., Olmos, D., Saunders, E., Leongamornlert, D., Tymrakiewicz, M., Mahmud, N., Dadaev, T., Govindasami, K., Guy, M., et al. (2013). Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer. J. Clin. Oncol. *31*, 1748–1757.

Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E., et al. (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. *2*, 401–404.

Cibulskis, K., Lawrence, M.S., Carter, S.L., Sivachenko, A., Jaffe, D., Sougnez, C., Gabriel, S., Meyerson, M., Lander, E.S., and Getz, G. (2013). Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat. Biotechnol. *31*, 213–219.

Comstock, C.E., Augello, M.A., Goodwin, J.F., de Leeuw, R., Schiewer, M.J., Ostrander, W.F., Jr., Burkhart, R.A., McClendon, A.K., McCue, P.A., Trabulsi, E.J., et al. (2013). Targeting cell cycle and hormone receptor pathways in cancer. Oncogene *32*, 5481–5491.

Cooper, C.S., Eeles, R., Wedge, D.C., Van Loo, P., Gundem, G., Alexandrov, L.B., Kremeyer, B., Butler, A., Lynch, A.G., Camacho, N., et al.; ICGC Prostate Group (2015). Analysis of the genetic phylogeny of multifocal prostate cancer identifies multiple independent clonal expansions in neoplastic and morphologically normal prostate tissue. Nat. Genet. *47*, 367–372.

de Bono, J.S., Logothetis, C.J., Molina, A., Fizazi, K., North, S., Chu, L., Chi, K.N., Jones, R.J., Goodman, O.B., Jr., Saad, F., et al.; COU-AA-301 Investigators (2011). Abiraterone and increased survival in metastatic prostate cancer. N. Engl. J. Med. *364*, 1995–2005.

Epstein, J.I., Amin, M.B., Beltran, H., Lotan, T.L., Mosquera, J.M., Reuter, V.E., Robinson, B.D., Troncoso, P., and Rubin, M.A. (2014). Proposed morphologic classification of prostate cancer with neuroendocrine differentiation. Am. J. Surg. Pathol. *38*, 756–767.

Finn, R.S., Crown, J.P., Lang, I., Boer, K., Bondarenko, I.M., Kulyk, S.O., Ettl, J., Patel, R., Pinter, T., Schmidt, M., et al. (2015). The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. Lancet Oncol. *16*, 25–35.

Fong, P.C., Boss, D.S., Yap, T.A., Tutt, A., Wu, P., Mergui-Roelvink, M., Mortimer, P., Swaisland, H., Lau, A., O'Connor, M.J., et al. (2009). Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N. Engl. J. Med. *361*, 123–134.

Futreal, P.A., Coin, L., Marshall, M., Down, T., Hubbard, T., Wooster, R., Rahman, N., and Stratton, M.R. (2004). A census of human cancer genes. Nat. Rev. Cancer *4*, 177–183.

Gao, J., Aksoy, B.A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S.O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., et al. (2013). Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci. Signal. *6*, pl1.

Gao, D., Vela, I., Sboner, A., Iaquinta, P.J., Karthaus, W.R., Gopalan, A., Dowling, C., Wanjala, J.N., Undvall, E.A., Arora, V.K., et al. (2014). Organoid cultures derived from patients with advanced prostate cancer. Cell *159*, 176–187.

Geng, C., He, B., Xu, L., Barbieri, C.E., Eedunuri, V.K., Chew, S.A., Zimmermann, M., Bond, R., Shou, J., Li, C., et al. (2013). Prostate cancer-associated

mutations in speckle-type POZ protein (SPOP) regulate steroid receptor coactivator 3 protein turnover. Proc. Natl. Acad. Sci. USA *110*, 6997–7002.

Giannakis, M., Hodis, E., Jasmine Mu, X., Yamauchi, M., Rosenbluh, J., Cibulskis, K., Saksena, G., Lawrence, M.S., Qian, Z.R., Nishihara, R., et al. (2014). RNF43 is frequently mutated in colorectal and endometrial cancers. Nat. Genet. *46*, 1264–1266.

Grasso, C.S., Wu, Y.M., Robinson, D.R., Cao, X., Dhanasekaran, S.M., Khan, A.P., Quist, M.J., Jing, X., Lonigro, R.J., Brenner, J.C., et al. (2012). The mutational landscape of lethal castration-resistant prostate cancer. Nature *487*, 239–243.

Gundem, G., Van Loo, P., Kremeyer, B., Alexandrov, L.B., Tubio, J.M., Papaemmanuil, E., Brewer, D.S., Kallio, H.M., Högnäs, G., Annala, M., et al.; ICGC Prostate UK Group (2015). The evolutionary history of lethal metastatic prostate cancer. Nature *520*, 353–357.

Hieronymus, H., Schultz, N., Gopalan, A., Carver, B.S., Chang, M.T., Xiao, Y., Heguy, A., Huberman, K., Bernstein, M., Assel, M., et al. (2014). Copy number alteration burden predicts prostate cancer relapse. Proc. Natl. Acad. Sci. USA *111*, 11139–11144.

Hong, M.K., Macintyre, G., Wedge, D.C., Van Loo, P., Patel, K., Lunke, S., Alexandrov, L.B., Sloggett, C., Cmero, M., Marass, F., et al. (2015). Tracking the origins and drivers of subclonal metastatic expansion in prostate cancer. Nat. Commun. *6*, 6605.

Hsieh, C.L., Botta, G., Gao, S., Li, T., Van Allen, E.M., Treacy, D.J., Cai, C., He, H.H., Sweeney, C.J., Brown, M., et al. (2015). PLZF, a tumor suppressor genetically lost in metastatic castration-resistant prostate cancer, is a mediator of resistance to androgen deprivation therapy. Cancer Res. Published online March 25, 2015. http://dx.doi.org/10.1158/0008-5472.CAN-14-3602.

Kaufman, B., Shapira-Frommer, R., Schmutzler, R.K., Audeh, M.W., Friedlander, M., Balmaña, J., Mitchell, G., Fried, G., Stemmer, S.M., Hubert, A., et al. (2015). Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. J. Clin. Oncol. *33*, 244–250.

Kikugawa, T., Kinugasa, Y., Shiraishi, K., Nanba, D., Nakashiro, K., Tanji, N., Yokoyama, M., and Higashiyama, S. (2006). PLZF regulates Pbx1 transcription and Pbx1-HoxC8 complex leads to androgen-independent prostate cancer proliferation. Prostate *66*, 1092–1099.

Kim, D., and Salzberg, S.L. (2011). TopHat-Fusion: an algorithm for discovery of novel fusion transcripts. Genome Biol. *12*, R72.

Lalonde, E., Ishkanian, A.S., Sykes, J., Fraser, M., Ross-Adams, H., Erho, N., Dunning, M.J., Halim, S., Lamb, A.D., Moon, N.C., et al. (2014). Tumour genomic and microenvironmental heterogeneity for integrated prediction of 5-year biochemical recurrence of prostate cancer: a retrospective cohort study. Lancet Oncol. *15*, 1521–1532.

Lawrence, M.S., Stojanov, P., Polak, P., Kryukov, G.V., Cibulskis, K., Sivachenko, A., Carter, S.L., Stewart, C., Mermel, C.H., Roberts, S.A., et al. (2013). Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature *499*, 214–218.

Lawrence, M.S., Stojanov, P., Mermel, C.H., Robinson, J.T., Garraway, L.A., Golub, T.R., Meyerson, M., Gabriel, S.B., Lander, E.S., and Getz, G. (2014). Discovery and saturation analysis of cancer genes across 21 tumour types. Nature *505*, 495–501.

Liu, J., Pan, S., Hsieh, M.H., Ng, N., Sun, F., Wang, T., Kasibhatla, S., Schuller, A.G., Li, A.G., Cheng, D., et al. (2013). Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. Proc. Natl. Acad. Sci. USA *110*, 20224–20229.

Lonigro, R.J., Grasso, C.S., Robinson, D.R., Jing, X., Wu, Y.M., Cao, X., Quist, M.J., Tomlins, S.A., Pienta, K.J., and Chinnaiyan, A.M. (2011). Detection of somatic copy number alterations in cancer using targeted exome capture sequencing. Neoplasia *13*, 1019–1025.

Mateo, J., Hall, E., Sandhu, S., Omlin, A., Miranda, S., Carreira, S., Goodall, J., Gillman, A., Mossop, H., Ralph, C., et al. (2014). Antitumour activity of the PARP inhibitor olaparib in unselected sporadic castration-resistant prostate cancer (CRPC) in the TOPARP trial. Annals Oncol. *25*, 1–41.

Mehra, R., Kumar-Sinha, C., Shankar, S., Lonigro, R.J., Jing, X., Philips, N.E., Siddiqui, J., Han, B., Cao, X., Smith, D.C., et al. (2011). Characterization of bone metastases from rapid autopsies of prostate cancer patients. Clin. Cancer Res. *17*, 3924–3932.

Olshen, A.B., Venkatraman, E.S., Lucito, R., and Wigler, M. (2004). Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics *5*, 557–572.

Palanisamy, N., Ateeq, B., Kalyana-Sundaram, S., Pflueger, D., Ramnarayanan, K., Shankar, S., Han, B., Cao, Q., Cao, X., Suleman, K., et al. (2010). Rearrangements of the RAF kinase pathway in prostate cancer, gastric cancer and melanoma. Nat. Med. *16*, 793–798.

Pflueger, D., Terry, S., Sboner, A., Habegger, L., Esgueva, R., Lin, P.C., Svensson, M.A., Kitabayashi, N., Moss, B.J., MacDonald, T.Y., et al. (2011). Discovery of non-ETS gene fusions in human prostate cancer using next-generation RNA sequencing. Genome Res. *21*, 56–67.

Pritchard, C.C., Morrissey, C., Kumar, A., Zhang, X., Smith, C., Coleman, I., Salipante, S.J., Milbank, J., Yu, M., Grady, W.M., et al. (2014). Complex MSH2 and MSH6 mutations in hypermutated microsatellite unstable advanced prostate cancer. Nat. Commun. *5*, 4988.

Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat. Biotechnol. *29*, 24–26.

Roychowdhury, S., Iyer, M.K., Robinson, D.R., Lonigro, R.J., Wu, Y.M., Cao, X., Kalyana-Sundaram, S., Sam, L., Balbin, O.A., Quist, M.J., et al. (2011). Personalized oncology through integrative high-throughput sequencing: a pilot study. Sci. Transl. Med. *3*, 111ra121.

Ryan, C.J., Smith, M.R., de Bono, J.S., Molina, A., Logothetis, C.J., de Souza, P., Fizazi, K., Mainwaring, P., Piulats, J.M., Ng, S., et al.; COU-AA-302 Investigators (2013). Abiraterone in metastatic prostate cancer without previous chemotherapy. N. Engl. J. Med. *368*, 138–148.

Scher, H.I., Fizazi, K., Saad, F., Taplin, M.E., Sternberg, C.N., Miller, K., de Wit, R., Mulders, P., Chi, K.N., Shore, N.D., et al.; AFFIRM Investigators (2012). Increased survival with enzalutamide in prostate cancer after chemotherapy. N. Engl. J. Med. *367*, 1187–1197.

Schwartz, S., Wongvipat, J., Trigwell, C.B., Hancox, U., Carver, B.S., Rodrik-Outmezguine, V., Will, M., Yellen, P., de Stanchina, E., Baselga, J., et al. (2015). Feedback suppression of PI3Kα signaling in PTEN-mutated tumors is relieved by selective inhibition of PI3Kβ. Cancer Cell *27*, 109–122.

Seshagiri, S., Stawiski, E.W., Durinck, S., Modrusan, Z., Storm, E.E., Conboy, C.B., Chaudhuri, S., Guan, Y., Janakiraman, V., Jaiswal, B.S., et al. (2012). Recurrent R-spondin fusions in colon cancer. Nature *488*, 660–664.

Taplin, M.E., Bubley, G.J., Shuster, T.D., Frantz, M.E., Spooner, A.E., Ogata, G.K., Keer, H.N., and Balk, S.P. (1995). Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N. Engl. J. Med. *332*, 1393–1398.

Taylor, B.S., Schultz, N., Hieronymus, H., Gopalan, A., Xiao, Y., Carver, B.S., Arora, V.K., Kaushik, P., Cerami, E., Reva, B., et al. (2010). Integrative genomic profiling of human prostate cancer. Cancer Cell *18*, 11–22.

The Cancer Genome Atlas. (2015). The molecular taxonomy of primary prostate cancer. http://www.cbioportal.org/study.do?cancer_study_id=prad_tcga_pub.

Thorvaldsdóttir, H., Robinson, J.T., and Mesirov, J.P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinform. *14*, 178–192.

Tomlins, S.A., Rhodes, D.R., Perner, S., Dhanasekaran, S.M., Mehra, R., Sun, X.W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., et al. (2005). Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science *310*, 644–648.

Tomlins, S.A., Laxman, B., Dhanasekaran, S.M., Helgeson, B.E., Cao, X., Morris, D.S., Menon, A., Jing, X., Cao, Q., Han, B., et al. (2007). Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature *448*, 595–599.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., and Pachter, L. (2012). Differential gene and

transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. *7*, 562–578.

Van Allen, E.M., Foye, A., Wagle, N., Kim, W., Carter, S.L., McKenna, A., Simko, J.P., Garraway, L.A., and Febbo, P.G. (2014a). Successful whole-exome sequencing from a prostate cancer bone metastasis biopsy. Prostate Cancer Prostatic Dis. *17*, 23–27.

Van Allen, E.M., Mouw, K.W., Kim, P., Iyer, G., Wagle, N., Al-Ahmadie, H., Zhu, C., Ostrovnaya, I., Kryukov, G.V., O'Connor, K.W., et al. (2014b). Somatic ERCC2 mutations correlate with cisplatin sensitivity in muscle-invasive urothelial carcinoma. Cancer Discov. *4*, 1140–1153.

Van Allen, E.M., Wagle, N., Stojanov, P., Perrin, D.L., Cibulskis, K., Marlow, S., Jane-Valbuena, J., Friedrich, D.C., Kryukov, G., Carter, S.L., et al. (2014c). Whole-exome sequencing and clinical interpretation of formalin-fixed, paraffin-embedded tumor samples to guide precision cancer medicine. Nat. Med. *20*, 682–688.

Voeller, H.J., Truica, C.I., and Gelmann, E.P. (1998). Beta-catenin mutations in human prostate cancer. Cancer Res. *58*, 2520–2523.

Wang, X.S., Shankar, S., Dhanasekaran, S.M., Ateeq, B., Sasaki, A.T., Jing, X., Robinson, D., Cao, Q., Prensner, J.R., Yocum, A.K., et al. (2011). Characterization of KRAS rearrangements in metastatic prostate cancer. Cancer Discov. *1*, 35–43.

Wee, S., Wiederschain, D., Maira, S.M., Loo, A., Miller, C., deBeaumont, R., Stegmeier, F., Yao, Y.M., and Lengauer, C. (2008). PTEN-deficient cancers depend on PIK3CB. Proc. Natl. Acad. Sci. USA *105*, 13057–13062.

Weston, V.J., Oldreive, C.E., Skowronska, A., Oscier, D.G., Pratt, G., Dyer, M.J., Smith, G., Powell, J.E., Rudzki, Z., Kearns, P., et al. (2010). The PARP inhibitor olaparib induces significant killing of ATM-deficient lymphoid tumor cells in vitro and in vivo. Blood *116*, 4578–4587.

The Journal of Molecular Diagnostics, Vol. 17, No. 6, November 2015

ELSEVIER

the Journal of Molecular Diagnostics
jmd.amjpathol.org

MSIplus for Integrated Colorectal Cancer Molecular Testing by Next-Generation Sequencing

Jennifer A. Hempelmann, Sheena M. Scroggins, Colin C. Pritchard, and Stephen J. Salipante

From the Department of Laboratory Medicine, University of Washington, Seattle, Washington

Accepted for publication May 26, 2015.

Address correspondence to Stephen J. Salipante, M.D., Ph.D., University of Washington, 1959 NE Pacific St, Room NW120, Box 357110, Seattle, WA 98195-7110. E-mail: stevesal@uw.edu.

Molecular analysis of colon cancers currently requires multiphasic testing that uses various assays with different performance characteristics, adding cost and time to patient care. We have developed a single, next-generation sequencing assay to simultaneously evaluate colorectal cancers for mutations in relevant cancer genes (*KRAS*, *NRAS*, and *BRAF*) and for tumor microsatellite instability (MSI). In a sample set of 61 cases, the assay demonstrated overall sensitivity of 100% and specificity of 100% for identifying cancer-associated mutations, with a practical limit of detection at 2% mutant allele fraction. MSIplus was 97% sensitive (34 of 35 MSI-positive cases) and 100% specific (42 of 42 MSI-negative cases) for ascertaining MSI phenotype in a cohort of 78 tumor specimens. These performance characteristics were slightly better than for conventional multiplex PCR MSI testing (97% sensitivity and 95% specificity), which is based on comparison of microsatellite loci amplified from tumor and matched normal material, applied to the same specimen cohort. Because the assay uses an amplicon sequencing approach, it is rapid and appropriate for specimens with limited available material or fragmented DNA. This integrated testing strategy offers several advantages over existing methods, including a lack of need for matched normal material, sensitive and unbiased detection of variants in target genes, and an automated analysis pipeline enabling principled and reproducible identification of cancer-associated mutations and MSI status simultaneously. *(J Mol Diagn 2015, 17: 705–714; http://dx.doi.org/10.1016/j.jmoldx.2015.05.008)*

After initial diagnosis, the molecular characterization of colorectal cancers may require several separate clinical tests. Evaluation of microsatellite instability (MSI) status is recommended testing on all primary colon cancers from patients 50 years or younger (and in older patients if specific pathological features are present[1]) to serve as a screening test for Lynch syndrome, a disease of hereditary cancer predisposition.[2,3] Moreover, MSI status provides diagnostic information about disease prognosis and predicted treatment response to fluorouracil,[3–6] and can, therefore, directly inform patient care. In the case of metastatic disease, additional molecular testing beyond MSI status is indicated. Recently updated guidelines from the National Comprehensive Cancer Network recommend that extended *NRAS* and *KRAS* testing is performed on all stage IV cancers[7] to identify mutations conferring resistance to epidermal growth factor receptor inhibitors.[8–10] If *RAS* gene mutational status is negative, testing for *BRAF* mutations is then also advised, given the poor response of *BRAF*-mutated tumors to cetuximab plus irinotecan, fluorouracil, and leucovorin combination therapy,[11] and also to epidermal growth factor receptor inhibitors used beyond first-line treatment.[12,13]

Independent molecular assays are currently used to test tumor specimens for MSI status and gene mutations. Molecular diagnosis of MSI is implemented using multiplexed PCR-based MSI testing (MSI-PCR), wherein a limited number of informative microsatellite markers[14,15] are PCR amplified from tumor and matched normal material, products are resolved using capillary gel electrophoresis, and the presence of additional alleles, which are the hallmark of the MSI

Supported by Congressionally Directed Medical Research Programs award PC131820 (C.C.P.) and a Prostate Cancer Foundation 2013 Young Investigator Award (C.C.P.).

Disclosures: None declared.

Copyright © 2015 American Society for Investigative Pathology and the Association for Molecular Pathology.
Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jmoldx.2015.05.008

phenotype, is qualitatively ascertained.[5,6,16] In some cases, immunohistochemical (IHC) detection of mismatch repair-pathway protein loss may additionally or alternatively be performed,[17–19] although some studies suggest that IHC does not sensitively identify all MSI-positive tumors.[17,20] In contrast, clinical testing for *NRAS*, *KRAS*, and *BRAF* gene mutations is commonly achieved using single-gene assays using melting curve analysis,[21,22] real-time PCR,[23,24] or conventional Sanger sequencing.[24] Peptide nucleic acid clamping[25] or selective amplification of mutant alleles is sometimes used to improve sensitivity.[26–28]

Next-generation sequencing (NGS) is becoming increasingly used by clinical laboratories as a cost-effective and scalable method to interrogate multiple genetic targets in parallel,[29–32] and could be adapted for the focused purpose of characterizing colorectal cancers for molecular workup. Although integrating colorectal cancer testing into an NGS diagnostic would offer practical advantages in eliminating the need for multiple, separate diagnostic tests, the unique analytic properties of NGS could also translate to performance advantages over existing testing methods. Such benefits include the following: i) The ability of the technology to detect low-prevalence, cancer-associated mutations is greater than that of conventional methods,[31–34] because each DNA molecule is examined independently by NGS, potentially providing increased sensitivity for detecting relevant cancer-associated mutations. ii) Our group[35] and others[36–38] have developed methods to computationally infer MSI status from NGS data on the basis of the quantification and distribution of observed allele lengths at microsatellite loci. This offers a standardized, statistical approach for interpreting MSI testing results, in contrast to the current practice of subjective interpretation of MSI-PCR electropherogram traces.[35] iii) Owing to the digital nature of NGS data, primary analysis can be readily automated,[31,35,39] ensuring consistency in test interpretation.

Herein, we describe the clinical validation of a novel NGS assay, MSIplus, for simultaneously evaluating tumor MSI status and mutational hotspots in *KRAS*, *NRAS*, and *BRAF* genes, and provide interpretive guidelines for its diagnostic use. In contrast to earlier research work, which has used NGS to evaluate MSI status from exome and targeted gene capture designs, our assay uses an amplicon sequencing approach, which enables effective targeting and high depth of coverage for the loci of interest. The assay is suitable for molecular characterization of colorectal cancers, does not require matched normal material for inference of MSI status, and is rapid, cost-effective, sensitive, and specific.

Materials and Methods

Selection of Target Sequences and Primer Design

For the purpose of inferring MSI status, we selected a panel of 11 microsatellite markers from our earlier analysis of colorectal cancer exome data[35] that were empirically found to be both most discriminatory for MSI and most frequently unstable in MSI-positive tumors (Table 1). We also incorporated the mononucleotide A/T tract of HSPH1,[40] the instability of which predicts sensitivity to particular anticancer agents, and the five microsatellite markers (MONO-27, BAT-25, BAT-26, NR-21, and NR-24) that compose a performance-enhanced derivative of the Bethesda panel[15] used in current clinical MSI-PCR assays.[16] Separate primers were designed to span exons containing relevant mutational hotspots in *KRAS* (exons 2, 3, and 4; codons 12, 13, 61, 117, and 147), *NRAS* (exons 2, 3, and 4; codons 12, 13, 61, 117, and 147), and *BRAF* (exon 15; codons 599, 600, and 601).

Multiplexed primer design was performed using MPprimer version 1.4[41] (*https://code.google.com/p/mpprimer*, last accessed October 15, 2013) with some manual curation. Genomic coordinates for each locus (human genome hg19/GRCh37) and PCR primer sequences are provided in Table 1. Primers were concatenated at the 5′ end to partial Illumina sequencing adaptors, which were extended in downstream steps. All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

Tumor Specimens and Clinical Testing

DNA from tumor specimens, which were predominantly colorectal cancers, but included a small subset of endometrial cancers, lung cancers, ovarian cancers, melanoma, and additional tumor types, was extracted from fresh-frozen tissue or formalin-fixed, paraffin-embedded tissue blocks. All tumors had >10% neoplastic cellularity, as estimated by review of hematoxylin and eosin–stained slides. Clinical specimens were obtained in accordance with the Declaration of Helsinki and the ethics guidelines of the human subjects division of the University of Washington (Seattle, WA).

Our study design is summarized in Figure 1. Clinical testing for mutations in *BRAF*, *KRAS*, and *NRAS* was performed in 61 specimens by the University of Washington Clinical Molecular Genetics Laboratory. Variants were identified using either the UW-OncoPlex NGS oncology assay[31] (*http://tests.labmed.washington.edu/UW-OncoPlex*, last accessed June 4, 2014) or PCR-amplification and melting curve analysis assays for each individual gene target.[21,22,26]

Clinical MSI-PCR testing of 81 colorectal tumor specimens was performed by the University of Washington Clinical Molecular Genetics Laboratory using the MSI analysis kit (Promega, Fitchburg, WI). Samples demonstrating instability at two or more of the five mononucleotide markers included in this panel were considered MSI positive [MSI high (MSI-H); diagnosis, 44 specimens]. All other specimens analyzed in this study did not demonstrate any unstable loci by MSI-PCR, and were considered MSI negative (microsatellite stable; diagnosis, 37 specimens). IHC staining for MLH1, MSH2, MSH6, and PMS2 was performed using standard diagnostic techniques.

Table 1 Loci and Primer Sequences

Assay stage	Target locus	Primer coordinates	Repeat type	Forward primer sequence	Reverse primer sequence
Stage 1 PCR	Bat-25	Chr4: 55598177-55598271	(A)22	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTGGAGGATGACGAGTTGGCCCTAGAC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCCCAAAGAGACAGCAGTTGGAACATGA-3′
Stage 1 PCR	Bat-26	Chr2: 47641524-47641622	(T)19	5′-ACACTCTTTCCCTACACGACGCTCTTCCG ATCTAGTGGAGTGGAGGAGGGGAGAGAAA-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCTTGCAGTTTCATCACTGTCTGCGGT-3′
Stage 1 PCR	MONO-27	Chr2: 39564859-39564957	(T)28	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTCTACTGTCCTACTGTGCCTGGCTCC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCCAGCCTGGGCAAGATAATGAGACCC-3′
Stage 1 PCR	NR-21	Chr14: 23652311-23652403	(A)22	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTCTGGTGCACAGAGCAGAACCATCCT-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCGCAACCTCAAAAGCTGCCTCCCTTT-3′
Stage 1 PCR	NR-24	Chr2: 95849327-95849419	(T)24	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTCTGTAGTCCCAGCTATTCGGGAGGC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCAAATGACCCCTTCCTGCCCATCACT-3′
Stage 1 PCR	MSI-01	Chr1: 201754376-201754446	(T)17	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTTTGATGTCCTGCGTCTAGGGTCTGC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCGACTGGAGCCTTGGACAGGTTGAGA-3′
Stage 1 PCR	MSI-03	Chr2: 62063059-62063129	(A)17	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTGCCACTGCTATTTGAAAGAGTTGCTC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCGCCACTGCTATTTGAAAGAGTTGCTC-3′
Stage 1 PCR	MSI-04	Chr2: 108479588-108479658	(T)18	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTTCCAAGATTCCTTCCCTGGCCACTC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCACTGTCTGTAGTCCTGGCTTCGTGG-3′
Stage 1 PCR	MSI-06	Chr5: 172421726-172421796	(T)15	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTAGCAGCAAACTGAACAGGTCACCAAC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCAGCAGCAAACTGAACAGGTCACCAAC-3′
Stage 1 PCR	MSI-07	Chr6: 142691916-142691986	(T)17	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTGCTGAAAGCAACCTAAGCTGTGGTGA-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCTGCTATAAGAGCTGAGCAGACGACA-3′
Stage 1 PCR	MSI-08	Chr7: 1787485-1787555	(A)17	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTCCAGCCCCCATGTACACTGTAGTCG-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCCCCACCCCAAGGCCAAAATCAGTAA-3′
Stage 1 PCR	MSI-09	Chr7: 74608706-74608776	(T)13	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTGTCTCGGCTACTTGGGAGGCTTAGG-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCCCGACTAAAGAGGTCATTCACTTGT-3′
Stage 1 PCR	MSI-11	Chr11: 106695477-106695550	(T)12	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTAGCATGTTTGCAGCCTTCTTCTGGA-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCAGCATGTTTGCAGCCTTCTTCTGGA-3′
Stage 1 PCR	MSI-12	Chr15: 45897737-45897807	(T)14	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTGCTGAGGCTAAACACTATCATGCCA-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCCAGAGGTTGCAGTGAGCCGAGATTG-3′
Stage 1 PCR	MSI-13	Chr16: 18882625-18882695	(A)15	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTACATCTTCAGGTCAGGAAAACAGCTCG-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCTAATGACTTGGGCTTTGGAAGCAGC-3′
Stage 1 PCR	MSI-14	Chr17: 19314883-19314953	(T)18	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTCATTTCAACTGACCTGCCTGGCCTC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCCTTGGCAAACGGGCAAGTCTTCAGT-3′
Stage 1 PCR	*HSPH1*-T17	Chr13: 31722570-31722746	(A)17	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTTGGAAAAGGAACTGCATCTGTGACGG-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCTTTTCCTAATCCCCTGTGAAACCTGT-3′
Stage 1 PCR	*BRAF* exon 15	Chr7: 140453095-140453431	NA	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTACAACTGTTCAAACTGATGGGACC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCCCTCATCCTAACACATTTCAAGCCCCA-3′
Stage 1 PCR	*KRAS* exon 4	Chr12: 25378395-25378686	NA	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTTTTCAGTGTTACTTACCTGTCTTGTC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCTGACAAAAGTTGTGGACAGGT-3′
Stage 1 PCR	*KRAS* exon 3	Chr12: 25380233-25380491	NA	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTCCCAGTCCTCATGTACTGGTCCCT-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCCCGTCATCTTTGGAGCAGGAACA-3′
Stage 1 PCR	*KRAS* exon 2	Chr12: 25398245-25398504	NA	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTTGAATTAGCTGTATCGTCAAGGCACTC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCACACGTCTGCAGTCAACTGGAATTT-3′
Stage 1 PCR	*NRAS* exon 4	Chr1: 115252168-115252401	NA	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTAATGCTGAAAGCTGTACCATACC-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCCCCAGCCTAATCTTGTTTTTCTT-3′
Stage 1 PCR	*NRAS* exon 3	Chr1: 115258629-115258838	NA	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTTGTGGCTCGCCAATTAACCCTG-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCTGAGAGACAGGATCAGGTCAGCGG-3′
Stage 1 PCR	*NRAS* exon 2	Chr1: 115256475-115256731	NA	5′-ACACTCTTTCCCTACACGACGCTCTTCCG-ATCTAGGAAGCCTTCGCCTGTCCTCA-3′	5′-CGGTCTCGGCATTCCTGCTGAACCGCTCTTGTG-TTTCACCAGATAGGCAGAAATGGGCTTGA-3′
Stage 2 PCR	NA	NA	NA	5′-AATGATACGGCGACCACCGAGATCTAC-ACTCTTTCCCTACACGACGC-3′	5′-CAAGCAGAAGACGGCATACGAGATXXXXXXXXCG-GTCTCGGCATTCCTGCTGAACCG-3′*
Index read	NA	NA	NA	5′-AGATCGGAAGAGCGGTTCAGCAGGA-ATGCCGCGCCCG-3′	NA

*X indicates the presence of an 8-bp sample-specific index sequence.
NA, not applicable.

Determination of mSINGS Baseline Reference Values

Determining MSI status by mSINGS analysis of NGS data[35] entails comparing experimental results against baseline reference values at each microsatellite locus to assess its instability. Because amplifying microsatellite loci by PCR generates a distribution of alternate fragments (stutter artifact) that results from template slippage during cycling,[42,43] it is necessary to establish assay-specific baseline values for each locus. To establish baseline reference values, we extracted DNA from 42 peripheral blood specimens and analyzed them using the MSIplus assay.

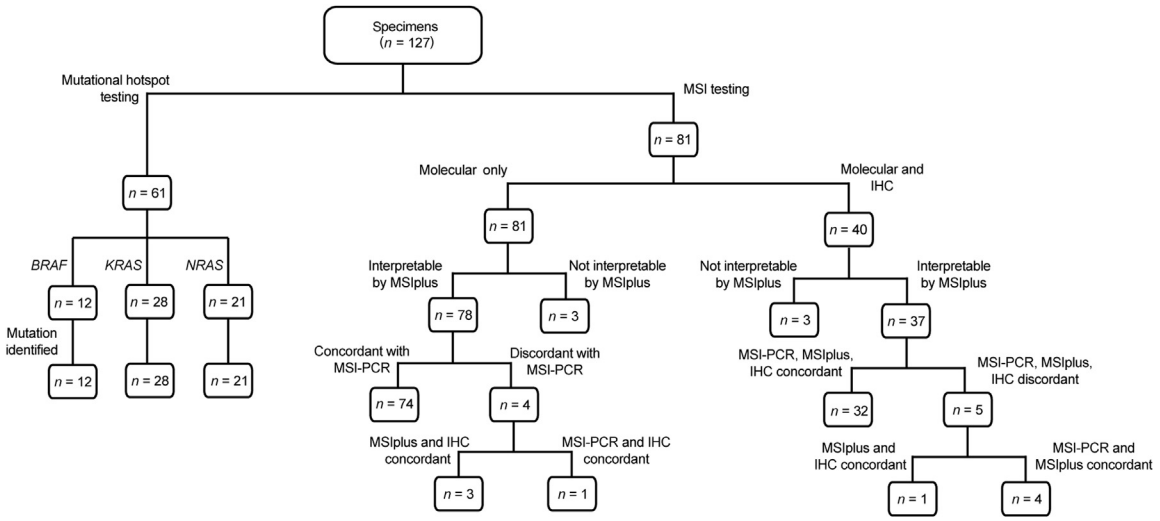


Figure 1 Study design and summary of results. Diagnostic testing algorithm and results are depicted. The number of specimens at each stage is indicated numerically at corresponding nodes. Fifteen specimens were tested for both microsatellite instability (MSI) status and mutational hotspots; thus, inclusion in these categories is not mutually exclusive. IHC, immunohistochemistry.

Library Preparation and Sequencing

Sequencing libraries were generated by PCR amplification in two separate stages. The purpose of the first stage was to simultaneously amplify the loci of interest from the genome and to incorporate partial Illumina sequencing adaptors into the amplification product. The second stage of PCR fully extended the sequencing adaptors and incorporated unique 8-bp, sample-specific index sequences (Table 1), which enabled the multiplexing of multiple specimens together onto the same sequencing run. In addition to the tumor samples, control DNA from HapMap individual NA12878 (Coriell Institute, Camden, NJ) was included with each library preparation and sequencing run, and served as a negative control for both MSI status and *RAS* and *BRAF* mutations, as was a nontemplate amplification control.

The first stage of PCR was performed in two separate reactions, one using an equimolar pool of the microsatellite primers, and the other using primers targeting mutational hotspots. In the latter primer pool, primers were combined in equimolar amounts, except for the primer pairs amplifying *NRAS* exon 2 (included at 0.5× concentration) and *NRAS* exon 4 (included at 1.5× concentration). Both first-stage PCRs were performed in a 25-μL volume using the Qiagen Multiplex PCR Kit (Qiagen, Valencia, CA), and incorporating 50-ng template DNA, 0.25 μmol/L of the appropriate primer pool, and 1× Qiagen Q-solution. PCR cycling for both primer pools was as follows: 5 minutes' incubation at 95°C; 30 cycles of 94°C for 30 seconds, 60°C for 90 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 10 minutes. Before the second stage of PCR, amplification products were purified using a 0.8× volume of Agencourt AMPure XP magnetic beads (Beckman-Coulter, Indianapolis, IN), according to the manufacturer's instructions.

The second stage of PCR was performed using 5 ng of amplification products from the first stage of PCR as template. PCR was performed in a 25-μL volume using KAPA HiFi HotStart ReadyMix PCR Kit (KAPABiosystems, Wilmington, MA), and 0.3 μmol/L of each of the second-stage primers (Table 1). This phase incorporated a reverse primer carrying a sample-specific index sequence; however, the same reverse primer was used in separately amplifying the microsatellite and mutational hotspot amplicons derived from the same specimen. PCR cycling conditions were as follows: 3 minutes' incubation at 95°C; five cycles of 98°C for 20 seconds, 65°C for 15 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 5 minutes. PCRs were purified using a 1.8× volume of Agencourt AMPure XP magnetic beads.

Amplicons derived from the same specimen were pooled in an 8:1 volumetric ratio of microsatellite PCR product/mutational hotspot PCR product before sequencing. Sequencing was performed on an Illumina MiSeq (San Diego, CA) using 200-bp, single-ended reads and an 8-bp index read, with the addition of a custom index read primer (Supplemental Table S1). A 5% concentration of PhiX Control version 3 (Illumina) was included in each sequencing run. Sequencing used a Micro or Nano MiSeq Reagent version 2 300-cycle kit (Illumina), depending on the number of samples pooled for sequencing (up to 32 samples and up to 9 samples, respectively).

Data Analysis

Single-ended sequence reads were initially aligned to the human genome (hg19/GRCh37) using bwa version 0.6.1-r104[44] (*http://sourceforge.net/projects/bio-bwa*, last accessed October 28, 2013) and SAMtools version 0.1.18 (*http://sourceforge.net/projects/samtools*, last accessed October 28, 2013).[45] Sample-level, fully local indel realignment was then

performed using Genome Analysis Toolkit version 3.2 (*https://www.broadinstitute.org/gatk*, last accessed May 28, 2014),[39,46] followed by quality score recalibration, to generate a final, realigned, and recalibrated alignment, which was used for subsequent analyses.

Identification of single-nucleotide variants, insertions, and deletions in *KRAS*, *NRAS*, and *BRAF* was performed using VarScan version 2.3.7 (*http://varscan.sourceforge.net*, last accessed May 16, 2014),[47] with the minimum variant frequency set to 0.005 reads, the minimum number of variant reads set to 2, and strand filtering disabled. In addition to primary variant calls, we also tabulated the absolute number of sequence reads matching specific variants of clinical actionability (Supplemental Table S2) using the VarScan readcounts function.

MSI status was determined using the mSINGS package (*https://bitbucket.org/uwlabmed/msings*, last accessed April 27, 2015)[35] with multiplier set to 1.75, msi_min_threshold set to 0.27, and msi_max_threshold set to 0.54.

Results

Sensitivity and Specificity for *KRAS*, *NRAS*, and *BRAF* Mutations

We evaluated the ability of MSIplus to detect mutations in *KRAS*, *NRAS*, and *BRAF* for a panel of 61 formalin-fixed, paraffin-embedded tumor specimens known to be positive for mutations in these genes on the basis of prior clinical testing (Table 2). We first estimated the frequency of false-positive sequence reads at each clinically significant site in *KRAS*, *NRAS*, and *BRAF* by tallying mutant reads (Supplemental Table S1) at known wild-type sites, on the basis of prior clinical testing. Of a total of 517,062 sequence reads examined from a subset of 27 specimens, 2127 sequence reads (0.4%) carried a false-positive mutation. Regardless, multiple reads must carry the same artifact mutation for a variant to be called; thus, this analysis overestimates false-positivity rate. We, therefore, considered each of the mutant calls independently, which yielded an average allele fraction of 0.07% (SD, 0.17%) for false-positive calls of any particular mutation. We set a minimum threshold of 2% allele fraction for calling mutations using MSIplus, a threshold that should exclude virtually all false-positive variant calls (z-score $= 1.5 \times 10^{-25}$) and that defines the practical limit of detection for this assay.

We next evaluated the assay's ability to detect clinically relevant mutations within the three target genes for each of 61 positive control specimens (Figure 1 and Table 2). Average read depth across the seven separate amplicons covering mutational hotspots was 1652 reads (interquartile range, 225 to 2306 reads). We achieved 100% sensitivity [61 of 61 expected variants recovered; 95% CI, 94.1%–100% by the Clopper-Pearson (exact binomial) method].

No specimen demonstrated a false-positive variant call occurring at or above a 2% allele fraction for any of the 44 possible nucleotide sequence variants encoding a clinically relevant mutation (Supplemental Table S1). These results equate to a specificity of 100% (2684 of 2684 true-negative variant calls; 95% CI, >99.3% to 100%). In addition, we observed a high degree of correlation ($R^2 = 0.84$) between the allele fraction of mutations detected by MSIplus and the estimated mutant allele fraction from previous clinical testing using targeted gene-capture NGS methods[31] (subset of 55 specimens) (Figure 2 and Supplemental Table S2). Approximate linearity between these estimates was observed over a range of 2.0% to 59.2% estimated variant allele fraction. This finding suggests that the amplification bias or other artifacts potentially affecting the calculated allele fraction are not pronounced in the MSIplus assay. Furthermore, variants in two specimens with 2.0% mutant allele frequencies were successfully identified, supporting the assay's theoretical limit of detection.

To evaluate the reproducibility of mutation detection, we examined a subset of eight control specimens across three or more independent batches of library preparation and sequencing (Supplemental Table S3). The expected mutations were recovered in all 36 independent technical replicates. The CV for the estimated allele fraction was 0.06 (range, 0.03 to 0.15).

Table 2 Detection of *KRAS*, *NRAS*, and *BRAF* Mutations

Mutation	No. of samples	No. detected
KRAS p.G12S	1	1
KRAS p.G12D	5	5
KRAS p.G12C	2	2
KRAS p.G12V	6	6
KRAS p.G13C	1	1
KRAS p.G13D	8	8
KRAS p.Q61H	3	3
KRAS p.K117N	1	1
KRAS p.A146V	1	1
NRAS p.G13V	1	1
NRAS p.G13R	1	1
NRAS p.G12S	1	1
NRAS p.G12D	6	6
NRAS p.Q61R	8	8
NRAS p.Q61L	2	2
NRAS p.Q61K	1	1
NRAS p.Q61H	1	1
BRAF p.V600K	1	1
BRAF p.V600E	11	11
Total	61	61

Determination of MSI Status

We separately assessed the assay's ability to detect the MSI-positive phenotype on the basis of mSINGS analysis of targeted microsatellite loci.[35] By using MSIplus, we typed a collection of 81 specimens (Figure 1) previously subjected to MSI-PCR testing in our laboratory (44 microsatellite unstable, or MSI-H, results and 37 microsatellite stable results). We first evaluated the reproducibility of mSINGS score determination (corresponding to the total

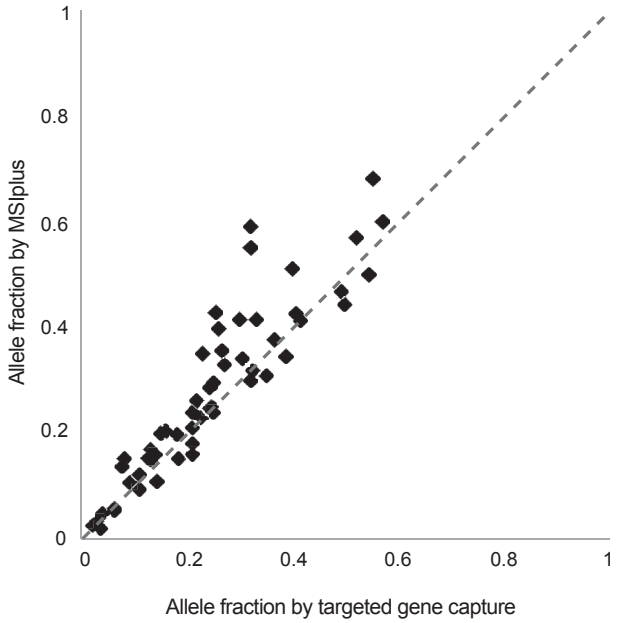


Figure 2 Correlation of allele fraction by MSIplus and targeted gene-capture next-generation sequencing. Allele fractions are estimated by either the MSIplus assay or the UW-OncoPlex targeted gene-capture sequencing panel. The subset of 55 specimens for which data from both assays were available is shown. Dashed gray line indicates a theoretical perfect correlation between the two estimations.

fraction of typed microsatellite loci that are unstable) by examining at least two separately prepared and sequenced technical replicates each for a subset of 48 specimens (Supplemental Table S4). Because several specimens had mSINGS scores of 0, calculating the CV was not meaningful. On average, the SD observed among technical replicates was 0.07 mSINGS units, and the SD of values around this mean was 0.03.

On the basis of the qualitative separation of microsatellite stable from MSI-H groups[35] (Figure 3), we initially set an empirical threshold of a 0.40 mSINGS score for differentiating MSI-positive from MSI-negative tumors. However, in light of mSINGS score variability, specimens with scores falling close to this threshold could be assigned the wrong MSI status by chance alone. To prevent improper MSI classifications resulting from assay variability, specimens with mSINGS values falling between 0.27 and 0.54 [threshold ± (average mSINGS variability + standard deviation of mSINGS variability × 2)] were consequently considered uninterpretable. In contrast, specimens with mSINGS scores lower or higher than those values could be confidently classified as MSI negative or MSI positive, respectively. Seventy-seven specimens had interpretable mSINGS scores, and we repeated library preparation for the remaining four samples that did not. On retyping, mSINGS scores for one of the four specimens became interpretable, whereas the remaining three results remained ambiguous. Thus, the MSI status of 95% of all specimens was initially interpretable, and on repeat typing, this proportion increased only modestly, to 96%.

Most MSI-negative specimens were readily distinguished from MSI-positive specimens on the basis of mSINGS score, and mSINGS interpretations were fully concordant with MSI-PCR interpretations in most cases (Figure 3 and Supplemental Table S5). However, four samples had discordant results between the two methods, which warranted further investigation.

One specimen was classified as MSI negative by MSI-PCR, but had a high mSINGS score (0.63) and was, therefore, interpreted as MSI positive by the MSIplus assay. Review of laboratory records indicated that, on subsequent workup, the tumor was found to have deficient MHS6 expression by IHC and that gene sequencing identified a germline MSH6 mutation in the patient, establishing a diagnosis of Lynch syndrome. We conclude that this instance represents a false-negative MSI-PCR result, and that the correct diagnosis was achieved by MSIplus. The other three discrepancies corresponded to specimens typed as MSI positive by MSI-PCR but classified as MSI negative using MSIplus. In two cases, clinical IHC testing was performed and did not reveal loss of expression in any mismatch repair proteins. Although MSI positivity has been observed in a background of normal MMR protein expression in approximately 5% of cases,[16] these cases are most consistent with false-positive MSI-PCR results. Conversely, the remaining discrepant case likely represents a false negative by MSIplus because loss of MSH2 and MSH6 expression was seen by IHC and a germline *MSH6* mutation was identified by genetic testing.

Accounting for these additional clinical data, and assuming that all specimens receiving a concordant

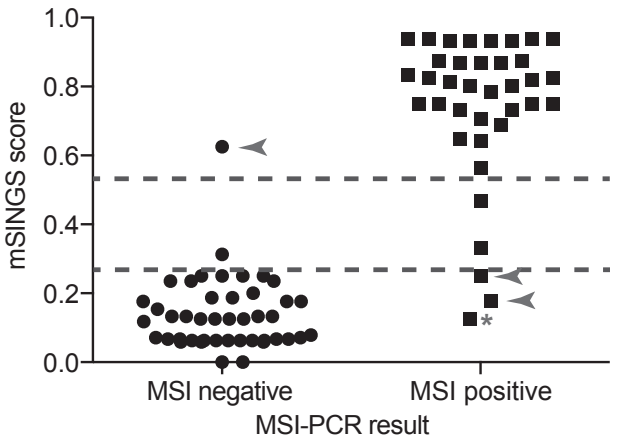


Figure 3 Inferring microsatellite instability (MSI) status by MSIplus. Data are stratified according to testing results by MSI-PCR. The mSINGS score (the fraction of interrogated microsatellite loci that are unstable) is plotted for each specimen. The dashed lines at mSINGS scores of 0.27 and 0.54 indicate the cutoffs for delineating MSI-positive and MSI-negative specimens by MSIplus: mSINGS scores falling below these values were interpreted as negative, scores above those values were interpreted as positive, and scores falling between the values could not be reliably interpreted. **Arrowheads** indicate specimens misclassified by MSI-PCR; **asterisk** indicates specimen misclassified by MSIplus, as resolved by alternative clinical testing results (immunohistochemical and/or genetic testing). For specimens that were typed multiple times, one representative mSINGS score is displayed.

diagnosis by MSI-PCR and MSIplus were correctly assigned an MSI status, we conclude that the validation set used in our study comprised a total of 36 MSI-positive cases and 42 MSI-negative cases. We calculate that MSIplus has an overall sensitivity of 97.1% (34 of 35 MSI-positive cases identified; 95% CI, 85.1%–99.9%) and a specificity of 100% (42 of 42 MSI-negative cases identified; 95% CI, 91.6%–100%) for determining tumor MSI status. The positive predictive value and negative predictive value for MSIplus were 97.1% and 97.7%, respectively. For the same panel of test specimens, MSI-PCR demonstrated a sensitivity of 97.1% (34 of 35 MSI-positive cases identified; 95% CI, 85.1%–99.9%), a specificity of 95.2% (40 of 42 MSI-negative cases identified; 95% CI, 83.8%–99.4%), a positive predictive value of 94.6%, and a negative predictive value of 95.6% for the same set of validation specimens.

Correlation of MSIplus, MSI-PCR, and IHC

Although our primary objective was to compare the results of molecular MSI testing by MSI-PCR and MSIplus, we additionally correlated our findings with IHC for 40 cases where this information was available (Figure 1 and Supplemental Table S5). The diagnosis rendered by each of the three approaches (IHC, MSI-PCR, and MSIplus) was fully concordant in 32 of 37 cases where MSIplus was interpretable. One discrepant case, discussed above, evidenced isolated loss of MSH6 by IHC, and was negative by MSI-PCR but positive by MSIplus, suggesting that MSIplus and IHC obtained the proper diagnosis. Three additional cases receiving a MSI-positive diagnosis by both MSI-PCR and MSIplus showed no loss of MMR protein expression by IHC, suggesting a MSI-positive status without loss of MMR protein expression.[20] One specimen was negative by MSI-PCR and MSIplus, but demonstrated reduction of MLH1 and PMS2 expression by IHC: it is possible that this latter case represents either a false-negative molecular result or a false-positive result by IHC.[20]

Quantitative Correlation of MSIplus and Conventional MSI-PCR

Last, we examined whether there was a correlation between the fraction of unstable markers characterized by MSIplus (ie, the mSINGS score) and the fraction of unstable microsatellite loci detected by MSI-PCR (Figure 4). We excluded from analysis specimens with discordant results between the two assays and those three having non-interpretable mSINGS scores.

Overall, the two measurements of MSI demonstrated a strong, positive correlation ($R^2 = 0.89$). Of 42 specimens with no unstable loci detectable by MSI-PCR, 20 demonstrated nonzero mSINGS scores; nevertheless, a trend of mSINGS overestimating the fraction of unstable loci was

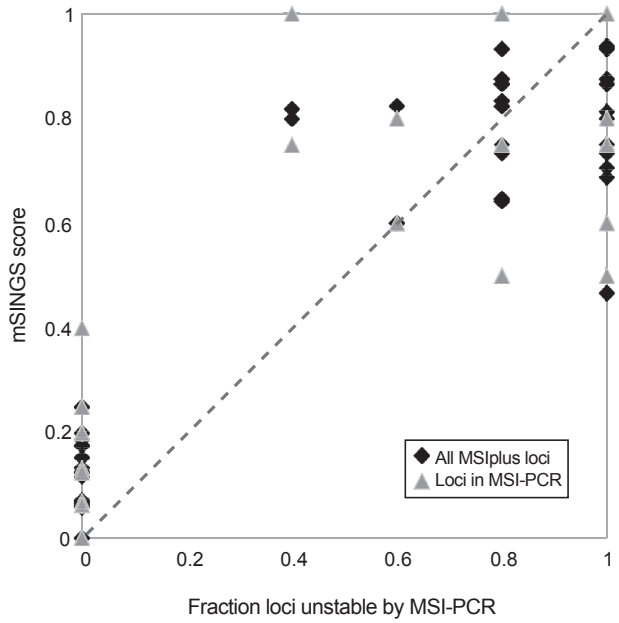


Figure 4 Correlation of locus instability measured by microsatellite instability (MSI)-PCR and MSIplus. The mSINGS score (the fraction of interrogated microsatellite loci that are unstable) is plotted against the fraction of unstable loci determined by MSI-PCR. Overall mSINGS score for the MSIplus assay is shown separately from mSINGS score calculated for the five loci in common with MSI-PCR. Dashed gray line indicates a theoretical perfect correlation between the two measures of instability.

not consistent across all specimens deemed MSI positive by MSI-PCR. We noted that if MSI-negative MSI-PCR results were removed from consideration, the correlation of mSINGS score and the fraction of unstable loci observed for MSI-H specimens was not statistically meaningful ($R^2 = 9.1 \times 10^{-3}$). This finding suggests that, above the threshold for delineating an MSI-positive phenotype, the fraction of unstable markers identified by MSI-PCR cannot be generalized to infer the degree of overall genomic instability.

We separately evaluated correlation between the fraction of unstable loci identified by either MSI-PCR or MSIplus for the subset of five loci that are represented in both assays. Again, a strong positive correlation was observed between these two metrics ($R^2 = 0.80$), although the lack of identity between them indicates subtle differences in individual loci being scored as stable or unstable between the two assays.

Discussion

Because the number of single-gene molecular tests needed to adequately characterize tumors continues to increase, the practical consequences of increased health care costs, increased test turnaround times, and the potential to deplete available tissue material during the course of testing become an increasing concern.[31] The use of highly scalable NGS technologies has proved a means to overcome this challenge, in many cases improving the quality and capabilities

of molecular testing.[30–33] In light of these considerations, we developed MSIplus, an NGS assay for characterizing colorectal tumor specimens that integrates extended *RAS* and *BRAF* gene testing with MSI analysis and, therefore, encompasses recommended molecular testing guidelines in a single assay.[7]

MSIplus had an overall sensitivity of 100% and a specificity of 100% for identifying cancer-associated mutations in *BRAF*, *KRAS,* and *NRAS* genes, as calculated from a panel of 61 specimens carrying a spectrum of known mutations (Table 2). The ability of a molecular assay to detect mutations present in a tumor specimen is dependent, in part, on the proportion of neoplastic cells in the sample and the fraction of total tumor cells that carry the mutation of interest.[31] The limit of detection for MSIplus is a 2% mutant allele fraction, lower than other prevalent clinical diagnostic methods.[21,22] Because the limit of detection in this assay partially reflects the rate of false-positive sequence reads, the sensitivity of MSIplus for low-prevalence mutations could potentially be improved in future iterations through practices such as incorporating molecular tagging-mediated sequencing error correction.[34,48,49] However, such methods present technical challenges and could negatively affect other aspects of the assay's performance.

Because it is a sequencing-based approach, MSIplus should be able to identify most single-nucleotide polymorphisms or small insertions or deletions occurring within the selected target regions. Compared with real-time PCR or melt-curve assays, which are designed to detect only a specific subset of actionable mutations,[23,24] MSIplus is capable of identifying variants without prior knowledge or expectation of the underlying genetic change. This property greatly improves the ability of MSIplus to identify rare or unusual clinically significant nucleotide alterations.

MSIplus had 97.1% sensitivity and 100% specificity for characterizing the MSI phenotype in a test set of 78 tumors, performance characteristics that were similar to those reported for MSI-PCR in other studies[5,16] and slightly better than the performance of MSI-PCR for the same set of specimens. Although MSIplus does not substantially improve sensitivity or specificity compared with MSI-PCR, it has important advantages over existing assays. Evaluation of MSI by mSINGS analysis eliminates the need for matched normal patient material,[35] which is typically required for conventional MSI-PCR, thereby expanding the scope of available patient specimens that can be successfully typed using MSIplus. The interpretation of MSIplus uses an automated analysis pipeline that is based on quantitative, descriptive statistics. This feature may improve the consistency of MSI diagnosis and reduce interlaboratory and intralaboratory variation.

We were unable to confidently call MSI status using MSIplus for a small subset of specimens (95% of specimens were interpretable with the first round of testing, whereas only 5% were not). Three of the four specimens with indeterminate mSINGS scores again yielded uninterpretable results after repeated testing, suggesting that the assay cannot confidently type MSI status for a small fraction of samples. Although the biological significance of these persistently indeterminate mSINGS scores, if any, is unclear,[50,51] studies examining larger numbers of microsatellite markers have suggested that such cases do not represent a distinct disease category of subtype of MSI.[35,38] These indeterminate specimens could potentially be resolved by increasing the number of microsatellite loci examined in the assay to enable more accurate assessment of the mSINGS score.[35]

Our study identified several cases where IHC and molecular testing were discordant, as expected.[17,20] It is known that isolated MSH6 deficiencies may result in false-negative MSI-PCR results,[52] and our sample cohort contained one such case. Unlike MSI-PCR, MSIplus identified this MSH6-deficient specimen as MSI positive, rendering a proper molecular diagnosis. Moreover, all indeterminate MSIplus results occurred in cases where IHC indicated reduced MMR protein expression, including two testing negative by MSI-PCR. Although anecdotal, these findings suggest an improved ability of MSIplus to identify MSH6-mutated specimens and improved sensitivity for detecting MMR pathway deficiencies compared with MSI-PCR. With further refinement, the MSIplus assay may offer significant performance advantages over MSI-PCR for such cases.

We anticipate that MSIplus will prove useful in characterizing colorectal cancers while potentially reducing operating costs and standardizing the interpretation of testing results. The assay is rapid, and compatible with a 2- or 3-day turnaround time: library preparation, approximately 8 hours; sequencer setup, approximately 1 hour; sequencing, approximately 8 hours; data analysis, approximately 2 hours per specimen on a four-processor machine, but scalable on larger computing systems to process multiple specimens simultaneously. Because the assay uses PCR-mediated library preparation, it requires minimal input DNA (50 ng) and should function even for partially degraded specimens. The modular nature of the multiplexed primer design will enable relatively straightforward expansion of the assay to additional molecular targets, as necessary, in response to future diagnostic requirements. The approach of focused, integrated NGS testing, tailored to a specific tumor type or diagnostic workflow, is a powerful paradigm that can be adapted to other clinical scenarios, and will become more common as NGS technologies are increasingly integrated into clinical laboratories.

Acknowledgments

We thank the Molecular Diagnostics laboratory staff for their help in identifying and obtaining specimens and Tina Lockwood (Seattle, WA) for helpful conversations.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2015.05.008*.

References

1. Jenkins MA, Hayashi S, O'Shea AM, Burgart LJ, Smyrk TC, Shimizu D, Waring PM, Ruszkiewicz AR, Pollett AF, Redston M, Barker MA, Baron JA, Casey GR, Dowty JG, Giles GG, Limburg P, Newcomb P, Young JP, Walsh MD, Thibodeau SN, Lindor NM, Lemarchand L, Gallinger S, Haile RW, Potter JD, Hopper JL, Jass JR; Colon Cancer Family Registry: Pathology features in Bethesda guidelines predict colorectal cancer microsatellite instability: a population-based study. Gastroenterology 2007, 133:48–56
2. Beamer LC, Grant ML, Espenschied CR, Blazer KR, Hampel HL, Weitzel JN, MacDonald DJ: Reflex immunohistochemistry and microsatellite instability testing of colorectal tumors for Lynch syndrome among US cancer programs and follow-up of abnormal results. J Clin Oncol 2012, 30:1058–1063
3. Vilar E, Gruber SB: Microsatellite instability in colorectal cancer: the stable evidence. Nat Rev Clin Oncol 2010, 7:153–162
4. Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM, Hamilton SR, Laurent-Puig P, Gryfe R, Shepherd LE, Tu D, Redston M, Gallinger S: Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med 2003, 349:247–257
5. Goel A, Nagasaka T, Hamelin R, Boland CR: An optimized pentaplex PCR for detecting DNA mismatch repair-deficient colorectal cancers. PLoS One 2010, 5:e9393
6. de la Chapelle A, Hampel H: Clinical relevance of microsatellite instability in colorectal cancer. J Clin Oncol 2010, 28:3380–3387
7. NCCN Guidelines(R) Updates. J Natl Compr Canc Netw 2013, 11: xxxii–xxxvi
8. Plesec TP, Hunt JL: KRAS mutation testing in colorectal cancer. Adv Anat Pathol 2009, 16:196–203
9. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, et al: Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 2010, 11:753–762
10. Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocakova I, Ruff P, Blasinska-Morawiec M, Smakal M, Canon JL, Rother M, Williams R, Rong A, Wiezorek J, Sidhu R, Patterson SD: Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med 2013, 369:1023–1034
11. Van Cutsem E, Kohne CH, Lang I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, Schlichting M, Zubel A, Celik I, Rougier P, Ciardiello F: Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. J Clin Oncol 2011, 29:2011–2019
12. Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, Bardelli A: Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J Clin Oncol 2008, 26: 5705–5712
13. Bokemeyer C, Van Cutsem E, Rougier P, Ciardiello F, Heeger S, Schlichting M, Celik I, Kohne CH: Addition of cetuximab to chemotherapy as first-line treatment for KRAS wild-type metastatic colorectal cancer: pooled analysis of the CRYSTAL and OPUS randomised clinical trials. Eur J Cancer 2012, 48:1466–1475
14. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S: A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998, 58:5248–5257
15. Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Ruschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT, Peltomaki P, Ramsey SD, Rodriguez-Bigas MA, Vasen HF, Hawk ET, Barrett JC, Freedman AN, Srivastava S: Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 2004, 96:261–268
16. Bacher JW, Flanagan LA, Smalley RL, Nassif NA, Burgart LJ, Halberg RB, Megid WM, Thibodeau SN: Development of a fluorescent multiplex assay for detection of MSI-High tumors. Dis Markers 2004, 20:237–250
17. Lindor NM, Burgart LJ, Leontovich O, Goldberg RM, Cunningham JM, Sargent DJ, Walsh-Vockley C, Petersen GM, Walsh MD, Leggett BA, Young JP, Barker MA, Jass JR, Hopper J, Gallinger S, Bapat B, Redston M, Thibodeau SN: Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. J Clin Oncol 2002, 20:1043–1048
18. Shia J: Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome, part I: the utility of immunohistochemistry. J Mol Diagn 2008, 10:293–300
19. Zhang L: Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome, part II: the utility of microsatellite instability testing. J Mol Diagn 2008, 10:301–307
20. Müller A, Giuffre G, Edmonston TB, Mathiak M, Roggendorf B, Heinmöller E, Brodegger T, Tuccari G, Mangold E, Buettner R, Rüschoff J; German HNPCC Consortium German Cancer Aid (Deutsche Krebshife): Challenges and pitfalls in HNPCC screening by microsatellite analysis and immunohistochemistry. J Mol Diagn 2004, 6:308–315
21. Ikenoue T, Hikiba Y, Kanai F, Ijichi H, Togo G, Ohta M, Watabe H, Yamaji Y, Okamoto M, Aragaki J, Matsumura M, Kawabe T, Omata M: Rapid detection of mutations in the BRAF gene using real-time polymerase chain reaction and melting curve analysis. Cancer Genet Cytogenet 2004, 149:68–71
22. Nikiforova MN, Lynch RA, Biddinger PW, Alexander EK, Dorn GW 2nd, Tallini G, Kroll TG, Nikiforov YE: RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. J Clin Endocrinol Metab 2003, 88:2318–2326
23. Sakai K, Yoneshige A, Ito A, Ueda Y, Kondo S, Nobumasa H, Fujita Y, Togashi Y, Terashima M, De Velasco MA, Tomida S, Nishio K: Performance of a novel KRAS mutation assay for formalin-fixed paraffin embedded tissues of colorectal cancer. Springerplus 2015, 4:7
24. Qu K, Pan Q, Zhang X, Rodriguez L, Zhang K, Li H, Ho A, Sanders H, Sferruzza A, Cheng SM, Nguyen D, Jones D, Waldman F: Detection of BRAF V600 mutations in metastatic melanoma: comparison of the Cobas 4800 and Sanger sequencing assays. J Mol Diagn 2013, 15:790–795
25. Oh JE, Lim HS, An CH, Jeong EG, Han JY, Lee SH, Yoo NJ: Detection of low-level KRAS mutations using PNA-mediated asymmetric PCR clamping and melting curve analysis with unlabeled probes. J Mol Diagn 2010, 12:418–424
26. Pritchard CC, Akagi L, Reddy PL, Joseph L, Tait JF: COLD-PCR enhanced melting curve analysis improves diagnostic accuracy for KRAS mutations in colorectal carcinoma. BMC Clin Pathol 2010, 10:6
27. Li J, Wang L, Mamon H, Kulke MH, Berbeco R, Makrigiorgos GM: Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. Nat Med 2008, 14: 579–584

28. Westwood M, van Asselt T, Ramaekers B, Whiting P, Joore M, Armstrong N, Noake C, Ross J, Severens J, Kleijnen J: KRAS mutation testing of tumours in adults with metastatic colorectal cancer: a systematic review and cost-effectiveness analysis. Health Technol Assess 2014, 18:1−132
29. Pritchard CC, Smith C, Salipante SJ, Lee MK, Thornton AM, Nord AS, Gulden C, Kupfer SS, Swisher EM, Bennett RL, Novetsky AP, Jarvik GP, Olopade OI, Goodfellow PJ, King MC, Tait JF, Walsh T: ColoSeq provides comprehensive lynch and polyposis syndrome mutational analysis using massively parallel sequencing. J Mol Diagn 2012, 14:357−366
30. Frampton GM, Fichtenholtz A, Otto GA, Wang K, Downing SR, He J, et al: Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing. Nat Biotechnol 2013, 31:1023−1031
31. Pritchard CC, Salipante SJ, Koehler K, Smith C, Scroggins S, Wood B, Wu D, Lee MK, Dintzis S, Adey A, Liu Y, Eaton KD, Martins R, Stricker K, Margolin KA, Hoffman N, Churpek JE, Tait JF, King MC, Walsh T: Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. J Mol Diagn 2014, 16:56−67
32. Cottrell CE, Al-Kateb H, Bredemeyer AJ, Duncavage EJ, Spencer DH, Abel HJ, Lockwood CM, Hagemann IS, O'Guin SM, Burcea LC, Sawyer CS, Oschwald DM, Stratman JL, Sher DA, Johnson MR, Brown JT, Cliften PF, George B, McIntosh LD, Shrivastava S, Nguyen TT, Payton JE, Watson MA, Crosby SD, Head RD, Mitra RD, Nagarajan R, Kulkarni S, Seibert K, Virgin HW 4th, Milbrandt J, Pfeifer JD: Validation of a next-generation sequencing assay for clinical molecular oncology. J Mol Diagn 2014, 16:89−105
33. Harismendy O, Schwab RB, Bao L, Olson J, Rozenzhak S, Kotsopoulos SK, Pond S, Crain B, Chee MS, Messer K, Link DR, Frazer KA: Detection of low prevalence somatic mutations in solid tumors with ultra-deep targeted sequencing. Genome Biol 2011, 12:R124
34. Hiatt JB, Pritchard CC, Salipante SJ, O'Roak BJ, Shendure J: Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. Genome Res 2013, 23:843−854
35. Salipante SJ, Scroggins SM, Hampel HL, Turner EH, Pritchard CC: Microsatellite instability detection by next generation sequencing. Clin Chem 2014, 60:1192−1199
36. Lu Y, Soong TD, Elemento O: A novel approach for characterizing microsatellite instability in cancer cells. PLoS One 2013, 8:e63056
37. Niu B, Ye K, Zhang Q, Lu C, Xie M, McLellan MD, Wendl MC, Ding L: MSIsensor: microsatellite instability detection using paired tumor-normal sequence data. Bioinformatics 2014, 30:1015−1016
38. Kim TM, Laird PW, Park PJ: The landscape of microsatellite instability in colorectal and endometrial cancer genomes. Cell 2013, 155:858−868
39. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA: The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010, 20:1297−1303
40. Dorard C, de Thonel A, Collura A, Marisa L, Svrcek M, Lagrange A, Jego G, Wanherdrick K, Joly AL, Buhard O, Gobbo J, Penard-Lacronique V, Zouali H, Tubacher E, Kirzin S, Selves J, Milano G, Etienne-Grimaldi MC, Bengrine-Lefevre L, Louvet C, Tournigand C, Lefevre JH, Parc Y, Tiret E, Flejou JF, Gaub MP, Garrido C, Duval A: Expression of a mutant HSP110 sensitizes colorectal cancer cells to chemotherapy and improves disease prognosis. Nat Med 2011, 17:1283−1289
41. Shen Z, Qu W, Wang W, Lu Y, Wu Y, Li Z, Hang X, Wang X, Zhao D, Zhang C: MPprimer: a program for reliable multiplex PCR primer design. BMC Bioinformatics 2010, 11:143
42. Clarke LA, Rebelo CS, Goncalves J, Boavida MG, Jordan P: PCR amplification introduces errors into mononucleotide and dinucleotide repeat sequences. Mol Pathol 2001, 54:351−353
43. Salipante SJ, Horwitz MS: Phylogenetic fate mapping. Proc Natl Acad Sci U S A 2006, 103:5448−5453
44. Li H, Durbin R: Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009, 25:1754−1760
45. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R; 1000 Genomes Project Data Processing Subgroup: The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009, 25:2078−2079
46. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, Philippakis AA, del Angel G, Rivas MA, Hanna M, McKenna A, Fennell TJ, Kernytsky AM, Sivachenko AY, Cibulskis K, Gabriel SB, Altshuler D, Daly MJ: A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 2011, 43:491−498
47. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, Miller CA, Mardis ER, Ding L, Wilson RK: VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res 2012, 22:568−576
48. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B: Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A 2011, 108:9530−9535
49. Schmitt MW, Kennedy SR, Salk JJ, Fox EJ, Hiatt JB, Loeb LA: Detection of ultra-rare mutations by next-generation sequencing. Proc Natl Acad Sci U S A 2012, 109:14508−14513
50. Pawlik TM, Raut CP, Rodriguez-Bigas MA: Colorectal carcinogenesis: MSI-H versus MSI-L. Dis Markers 2004, 20:199−206
51. Tomlinson I, Halford S, Aaltonen L, Hawkins N, Ward R: Does MSI-low exist? J Pathol 2002, 197:6−13
52. Hampel H, Frankel W, Panescu J, Lockman J, Sotamaa K, Fix D, Comeras I, La Jeunesse J, Nakagawa H, Westman JA, Prior TW, Clendenning M, Penzone P, Lombardi J, Dunn P, Cohn DE, Copeland L, Eaton L, Fowler J, Lewandowski G, Vaccarello L, Bell J, Reid G, de la Chapelle A: Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients. Cancer Res 2006, 66:7810−7817

available at www.sciencedirect.com
journal homepage: www.europeanurology.com

European Association of Urology

Platinum Priority – Case Series of the Month

Editorial by Helen E. Bryant on pp. 996–997 of this issue

Biallelic Inactivation of *BRCA2* in Platinum-sensitive Metastatic Castration-resistant Prostate Cancer

Heather H. Cheng [a,b], ***Colin C. Pritchard*** [c], ***Thomas Boyd*** [d], ***Peter S. Nelson*** [e], ***Bruce Montgomery*** [a,*]

[a] *Department of Medicine, Division of Oncology, University of Washington, Seattle, WA, USA;* [b] *Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA;* [c] *Department of Laboratory Medicine, University of Washington, Seattle, WA, USA;* [d] *Northstar Lodge Cancer Center, Yakima, WA, USA;* [e] *Human Biology Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA*

Article info

Article history:
Accepted November 19, 2015

Associate Editor:
James Catto

Keywords:
BRCA2
Platinum
Carboplatin
Prostate cancer
mCRPC
DNA repair

Abstract

Understanding the molecular underpinnings of sensitivity to specific therapies will advance the goal of precision medicine in prostate cancer (PCa). We identified three patients with metastatic castration-resistant PCa (mCRPC) who achieved an exceptional response to platinum chemotherapy (not first-line treatment for PCa), despite disease progression on prior standard therapies. Using targeted next-generation sequencing on the primary and metastatic tumors, we found that all three patients had biallelic inactivation of *BRCA2*, a tumor suppressor gene critical for homologous DNA repair. Notably, two had germline *BRCA2* mutations, including a patient without compelling family history who was diagnosed at age 66 yr. The third patient had somatic *BRCA2* homozygous copy loss. Biallelic *BRCA2* inactivation in mCRPC warrants further exploration as a predictive biomarker for sensitivity to platinum chemotherapy.

© 2015 European Association of Urology. Published by Elsevier B.V. All rights reserved.

EU ACME
www.eu-acme.org/
europeanurology

* Corresponding author. Department of Medicine, Division of Oncology, University of Washington Medical Center, 1959 NE Pacific Street, Seattle, WA, 98195, USA.
E-mail address: rbmontgo@uw.edu (B. Montgomery).

1. Case report

Treatment for metastatic castration-resistant prostate cancer (mCRPC) now includes taxanes, androgen receptor pathway inhibitors, active cellular therapy, and a bone-targeting radiopharmaceutical [1]. Predictive biomarkers are needed to guide treatment selection and sequence. Reports describing the mutational landscape of mCRPC hold great promise for precision medicine, but actionable treatment decisions remain unclear [2–4].

Platinum chemotherapy is infrequently used for prostate cancer (PCa) except in cases of neuroendocrine differentiation [5]. We identified three patients with non-neuroendocrine mCRPC with an exceptional response to platinum, defined as patients with advanced cancer who attain a complete or partial response lasting at least 6 mo when expected response is $\leq$20%. To identify molecular changes associated with exceptional response, we retrospectively performed clinical targeted next-generation sequencing on tumor DNA. Surprisingly, the common finding between all three was biallelic inactivation of *BRCA2*, the homologous recombination DNA repair gene.

Patient 1 was diagnosed at age 66 yr with prostate-specific antigen (PSA) of 24.8 ng/ml and Gleason 4 + 4 prostate

http://dx.doi.org/10.1016/j.eururo.2015.11.022
0302-2838/© 2015 European Association of Urology. Published by Elsevier B.V. All rights reserved.

CrossMark

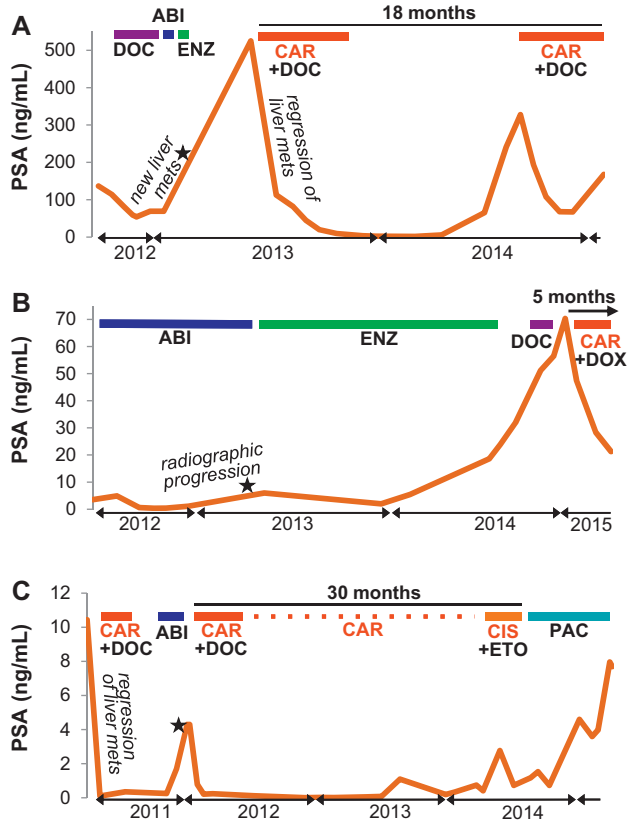


Fig. 1 – Clinical treatment course and prostate-specific antigen response. (A) Patient 1, (B) patient 2, (C) patient 3. Platinum chemotherapies are in bold red type. Stars denote time of metastatic biopsies. ABI = abiraterone; CAR = carboplatin; CIS = cisplatin; DOC = docetaxel; DOX = doxorubicin; ENZ = enzalutamide; ETO = etoposide; mets = metastases; PAC = paclitaxel; PSA = prostate-specific antigen.

adenocarcinoma and underwent neoadjuvant androgen deprivation therapy (ADT) on a clinical study followed by radical prostatectomy and salvage radiotherapy. After 4 yr, he was found to have metastases to the liver, lymph nodes, and bone. Biopsy of liver metastases revealed adenocarcinoma without evidence of neuroendocrine differentiation.

He received docetaxel with a PSA decline from 136 to 59 ng/ml, followed by PSA rise and then treatment with abiraterone followed by enzalutamide, both resulting in PSA and radiographic progression. Given earlier progression on docetaxel alone, he then received docetaxel and carboplatin with a PSA decline (Fig. 1A) and radiographic response (not shown). After a 6-mo break from treatment, he resumed docetaxel/carboplatin, again, with PSA decline. Unfortunately, he developed worsening malignant pleural effusions and ascites and ultimately elected to transition to hospice.

DNA sequencing of the metastatic liver biopsy revealed two *BRCA2* mutations, p.Q3066X and an exon 11 partial deletion on separate alleles (Table 1).

Patient 1 did not have a significant family history of cancer despite clear evidence of an inherited deleterious *BRCA2* mutation on germline testing. In light of these findings, he was referred to the medical genetics department, and mutation was confirmed.

Patient 2 was diagnosed at age 53 yr with PSA of 6.8 ng/ml and Gleason 5 + 4 prostate adenocarcinoma and underwent radical prostatectomy followed by salvage radiotherapy with ADT. After 5 yr, his PSA had risen to 12.0 ng/ml, and bone metastases were identified. He received 3 yr of intermittent ADT. On developing castration resistance, he was treated with abiraterone and then enzalutamide; both resulted in transient control and then PSA rise (Fig. 1B) and progressive disease. He then received docetaxel with no response, followed by carboplatin/doxorubicin for 6 mo with PSA and clinical response. Sequencing of a metastatic biopsy identified two deleterious *BRCA2* frameshift mutations including one that was germline (Table 1).

Patient 2 had a family history suggestive of a high-penetrance germline mutation with both father and paternal grandfather with PCa and a paternal aunt with breast cancer. He was referred to the medical genetics department, and mutation was confirmed.

Patient 3 was diagnosed at age 70 yr with PSA of 4.9 ng/ml and Gleason 5 + 5 prostate adenocarcinoma metastatic to pelvic and retroperitoneal lymph nodes. He developed castration resistance 6 mo after initiating ADT and was found to have liver metastases when PSA reached 10.0 ng/ml. Metastatic liver biopsy was obtained to assess for neuroendocrine differentiation, which was ruled out with immunohistochemistry. He was treated with docetaxel/carboplatin with a near-complete radiographic and PSA response (Fig. 1C). He was subsequently treated with abiraterone with disease progression and went on to receive a second course of docetaxel/carboplatin with another near-complete radiographic and PSA response. He then received "maintenance" carboplatin for >2 yr of progression-free survival. Sequencing of the previously obtained liver biopsy revealed somatic homozygous *BRCA2* copy loss in the metastasis (Table 1, Fig. 2). We were unable to confirm the

Table 1 – *BRCA2* mutations identified

		BRCA2 mutations[†]	Mutation type	Germline	Primary	Metastases
Patient 1	Allele 1	**c.9196C>T; p.Q3066X**	Premature stop	X	X	X
	Allele 2	**127bp del in exon 11**	Frameshift deletion		X	X
Patient 2	Allele 1	**c.8904delC; p.V2969Cfs*7**	Frameshift deletion	X	X	X
	Allele 2	**c.2611delT; p.S871Qfs*3**	Frameshift deletion		[‡]	X
Patient 3	Allele 1	**Homozygous copy loss**	Copy loss		X	X
	Allele 2	**Homozygous copy loss**	Copy loss		X	X

[†] Mutations are reported using reference transcript NM 000059.3.
[‡] Low tumor purity limited detection of somatic mutations.

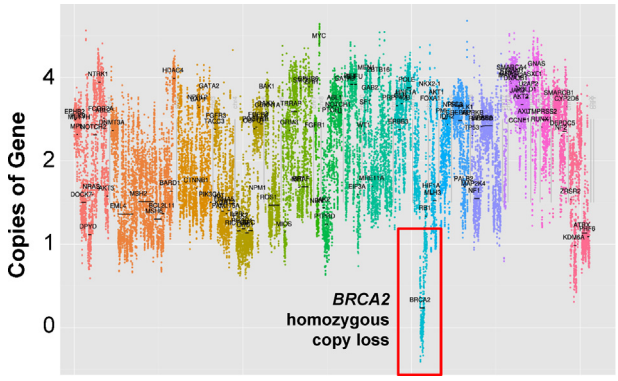


Fig. 2 – Copy number variation plot for metastatic tumor from patient 3. Changes in number of copies of genes within tumor cells were identified directly by UW-OncoPlex sequencing: (*x*-axis, left to right) each chromosome (1–22, x) is depicted in a different color; (*y*-axis) copies of genes. Selected genes are labeled.

presence of biallelic *BRCA2* copy loss in the primary tumor due to insufficient tumor.

2. Discussion

This case series documents three patients with mCRPC who achieved exceptional clinical response to platinum-based therapy after failure or progression on first-line therapies. Using a targeted next-generation sequencing clinical assay, we identified that all three had biallelic *BRCA2* inactivation in their tumors through either homozygous copy loss or germline deleterious mutation and somatic loss of function in the second allele in their metastases. By selecting for tumors with hypersensitivity to DNA damage induced by platinum chemotherapies, it is biologically plausible that defects in DNA repair would be revealed, particularly in light of observed platinum sensitivity in *BRCA1/2* germline mutation carriers in other cancer types, notably ovarian and breast cancers [6].

The prevalence of inactivation of *BRCA2* and other DNA damage repair genes in mCRPC is higher than previously thought and will require further validation. In one report, 7 of 50 (14%) patients with lethal PCa were found to have alterations in *BRCA2* [2]. The whole-exome sequencing results of the initial 150 mCRPC metastases from the SU2C Prostate International Dream Team demonstrated that 19% had aberrations in DNA repair genes (a combination of somatic and germline) including *BRCA1*, *BRCA2*, and *ATM* [4].

Inheritance of deleterious *BRCA2* mutations is well established to increase the risk of developing PCa in addition to breast, ovarian, pancreas, and other malignancies. Male *BRCA2* mutation carriers with localized PCa are at substantially higher risk of dying from PCa than their non–mutation-carrying counterparts [7]. Together with the SU2C data, this suggests that biallelic inactivation (germline and somatic) of *BRCA2* and related homologous recombination genes may be enriched among patients with aggressive mCRPC, especially when compared with the broader population of all (mostly indolent) PCa.

Our case series provides evidence that homozygous inactivation of *BRCA2* in mCRPC may confer sensitivity to platinum agents. Our series is limited by its small numbers and retrospective nature, but it suggests that inactivation of *BRCA2* and other DNA repair genes could be clinically useful as predictive biomarkers of platinum response. Whether other patients with hemizygous or homozygous inactivation of *BRCA2* or those with inactivation of other DNA repair pathway genes will be sensitive to DNA-damaging agents can only be addressed in prospective studies.

To our knowledge, this report is the first to associate dramatic response to platinum in men who had mCRPC and who were unselected for *a priori* mutation carriage with biallelic loss of *BRCA2*. Our report adds substantively to prior case reports that known *BRCA2* mutation carriers with mCRPC may respond particularly well to platinum chemotherapies. This mirrors breast and ovarian cancers, in which platinum chemotherapies are commonly used, and evidence suggests that germline and somatic mutations in homologous recombination genes such as *BRCA2* are associated with response to platinum and overall survival [8].

BRCA1/2 mutation carriers have also been effectively treated with a poly (ADP-ribose) polymerase inhibitor (PARPi) that renders synthetic lethality in cells with defective homologous DNA repair. Dramatic responses to PARPi have been reported very recently by Mateo et al [9]. Among men with mCRPC no longer responding to standard therapies whose tumors had evidence of DNA repair defects (including *BRCA2, ATM*, Fanconi anemia genes, and *CHEK2*), treatment with the PARPi olaparib resulted in a response rate of 88% (14 of 16) [9]. Clinical studies testing platinum agents, in combination and/or in sequence with PARPi, should also be explored for the subset of mCRPC patients whose tumors have biallelic inactivation of *BRCA2* and related homologous recombination repair pathway genes.

In the context of emerging data that *BRCA1/2* mutations may be present in up to 20% of mCRPC [4] and that *BRCA2* mutation–associated PCa is more aggressive [7], we are heartened by the dramatic platinum responses in these three patients whose tumors carried biallelic inactivation of *BRCA2*. Collectively, recent findings present a strong case for larger studies evaluating the tumors of all men who develop metastatic PCa for biallelic inactivation of *BRCA2* and related homologous DNA repair genes. These appear to be likely predictive biomarkers for treatment response to DNA-damaging therapy such as PARP inhibition and the widely available platinum chemotherapies.

3. Methods

We identified 14 patients with mCRPC treated with docetaxel and carboplatin between 2010 and the present. Although there was no standard institutional approach to treating patients with docetaxel and carboplatin, none had evidence of neuroendocrine differentiation, and most had aggressive features such as visceral involvement. Overall, 5 of 14 patients (36%) achieved treatment response, defined as PSA decline by 50% or radiographic partial response. Three patients had tumors available for analysis and are reported here. All three patients provided written informed consent for review of their medical record and sequencing

of their primary and metastatic PCa tissue. Research was conducted with University of Washington institutional review board approval.

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) samples, as previously described [10]. Slides stained with hematoxylin and eosin were reviewed before DNA extraction for all FFPE samples, and when feasible, macrodissection of tumor areas was performed to enrich tumor cellularity. We performed sequencing with UW-OncoPlex (University of Washington, Seattle, WA, USA), a validated clinical molecular diagnostic assay that collects simultaneous deep-sequencing information, based on >500 times average coverage, for all classes of mutations in 194 targeted clinically relevant genes, as previously reported [10]. At the time of this writing, 31 patients with PCa have undergone tumor sequencing with UW-OncoPlex at our institution. Four of 31 (13%) were identified to have biallelic *BRCA2* inactivation, 1 of whom died before results became available and without receiving platinum chemotherapy. The cases of the three others were reported in this paper.

Conflicts of interest: The authors have nothing to disclose.

Funding/Support and role of the sponsor: This work was supported by generous funding from the Institute for Prostate Cancer Research, the Pacific Northwest Prostate Cancer SPORE CA097186, 2013 Young Investigator Award from the Prostate Cancer Foundation (CCP), and Congressional Designated Medical Research Program (CDMRP) award PC131820 (CCP).

EU-ACME question

Please visit www.eu-acme.org/europeanurology to answer the following EU-ACME question online (the EU-ACME credits will be attributed automatically).

Question:

In addition to the potential association of biallelic inactivation of *BRCA2* with sensitivity of metastatic castration-resistant prostate cancer to platinum chemotherapy reported in this case series, there is evidence in the literature to support the following statements:

A. When an inactivating *BRCA2* mutation is identified in germline DNA, there may be an increased risk of prostate cancer in addition to breast, ovarian, and pancreatic cancers.
B. There is a higher risk of prostate cancer–associated death among prostate cancer patients who are *BRCA2* mutation carriers compared with noncarriers.
C. Recent data suggest that up to 20% of metastatic castration-resistant prostate cancers may contain biallelic inactivation of DNA damage repair genes such as *BRCA2*.
D. All of the above.

References

[1] Basch E, Loblaw DA, Oliver TK, et al. Systemic therapy in men with metastatic castration-resistant prostate cancer. American Society of Clinical Oncology and Cancer Care Ontario clinical practice guideline. J Clin Oncol 2014;32:3436–48.
[2] Grasso CS, Wu YM, Robinson DR, et al. The mutational landscape of lethal castration-resistant prostate cancer. Nature 2012;487:239–43.
[3] Beltran H, Yelensky R, Frampton GM, et al. Targeted next-generation sequencing of advanced prostate cancer identifies potential therapeutic targets and disease heterogeneity. Eur Urol 2013;63:920–6.
[4] Robinson D, Van Allen EM, Wu YM, et al. Integrative clinical genomics of advanced prostate cancer. Cell 2015;161:1215–28.
[5] Beltran H, Tomlins S, Aparicio A, et al. Aggressive variants of castration-resistant prostate cancer. Clin Cancer Res 2014;20:2846–50.
[6] Tan DS, Rothermundt C, Thomas K, et al. "BRCAness" syndrome in ovarian cancer: a case-control study describing the clinical features and outcome of patients with epithelial ovarian cancer associated with BRCA1 and BRCA2 mutations. J Clin Oncol 2008;26:5530–6.
[7] Castro E, Goh C, Leongamornlert D, et al. Effect of BRCA mutations on metastatic relapse and cause-specific survival after radical treatment for localised prostate cancer. Eur Urol 2015;68:186–93.
[8] Scott CL, Swisher EM, Kaufmann SH. Poly (ADP-ribose) polymerase inhibitors: recent advances and future development. J Clin Oncol 2015;33:1397–406.
[9] Mateo J, Carreira S, Sandhu S, et al. DNA-repair defects and olaparib in metastatic prostate cancer. N Engl J Med 2015;373:1697–708.
[10] Pritchard CC, Salipante SJ, Koehler K, et al. Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. J Mol Diagn 2014;16:56–67.

A Pilot Study of Clinical Targeted Next Generation Sequencing for Prostate Cancer: Consequences for Treatment and Genetic Counseling

Heather H. Cheng,[1,2]* Nola Klemfuss,[3] Bruce Montgomery,[1,2] Celestia S. Higano,[1] Michael T. Schweizer,[1,2] Elahe A. Mostaghel,[1,2] Lisa G. McFerrin,[3] Evan Y. Yu,[1,2] Peter S. Nelson,[1,3] and Colin C. Pritchard[4]

[1]*Division of Oncology, Department of Medicine, University of Washington, Seattle, Washington*
[2]*Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington*
[3]*Human Biology Division, Fred Hutchinson Cancer Research Center, Seattle, Washington*
[4]*Department of Laboratory Medicine, University of Washington, Seattle, Washington*

BACKGROUND. Targeted next generation sequencing (tNGS) is increasingly used in oncology for therapeutic decision-making, but is not yet widely used for prostate cancer. The objective of this study was to determine current clinical utility of tNGS for prostate cancer management.
METHODS. Seven academic genitourinary medical oncologists recruited and consented patients with prostate cancer, largely with unusual clinical and/or pathologic features, from 2013 to 2015. UW-OncoPlex was performed on formalin-fixed, paraffin-embedded (FFPE) primary tumors and/or metastatic biopsies. Results were discussed at a multidisciplinary precision tumor board prior to communicating to patients. FFPE tumor DNA was extracted for tNGS analysis of 194 cancer-associated genes. Results, multidisciplinary discussion, and treatment changes were recorded.
RESULTS. Forty-five patients consented and 42 had reportable results. Findings included mutations in genes frequently observed in prostate cancer. We also found alterations in genes where targeted treatments were available and/or in clinical trials. 4/42 (10%) cases, change in treatment directly resulted from tNGS and multidisciplinary discussion. In 30/42 (71%) cases additional options were available but not pursued and/or were pending. Notably, 10/42 (24%) of patients harbored suspected germline mutations in moderate or high-penetrance cancer risk genes, including *BRCA2*, *TP53*, *ATM*, and *CHEK2*. One patient's tumor had bi-allelic *MSH6* mutation and microsatellite instability. In total, 34/42 (81%) cases resulted in some measure of treatment actionability. Limitations include small size and limited clinical outcomes.
CONCLUSIONS. Targeted NGS tumor sequencing may help guide immediate and future treatment options for men with prostate cancer. A substantial subset had germline mutations in cancer predisposition genes with potential clinical management implications for men and their relatives. *Prostate 9999: 1–9, 2016.* © 2016 Wiley Periodicals, Inc.

KEY WORDS: **targeted next generation sequencing; panel testing; NGS; prostate cancer; precision tumor board**

Grant sponsor: Institute for Prostate Cancer Research; Grant sponsor: Pacific Northwest Prostate Cancer SPORE; Grant number: CA097186; Grant sponsor: Young Investigator Awards from the Prostate Cancer Foundation (HHC, MTS, CCP); Grant sponsor: Congressional Designated Medical Research Program (CDMRP); Grant number: PC131820 (CCP).

The authors declare no potential conflicts of interest.

*Correspondence to: Heather H. Cheng, MD, PhD, Division of Oncology, Department of Medicine, University of Washington, 825 Eastlake Ave E, Seattle, WA 98109. E-mail: hhcheng@uw.edu
Received 28 April 2016; Accepted 1 June 2016
DOI 10.1002/pros.23219
Published online in Wiley Online Library
(wileyonlinelibrary.com).

© 2016 Wiley Periodicals, Inc.

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer and the second most common cause of cancer death among men in the U.S. Many treatments are now available for advanced disease including docetaxel, sipuleucel-T, cabazitaxel, abiraterone, enzalutamide, and radium-223 with more in pre-clinical and clinical development [1]. With the growing number of effective treatments with survival benefit, the need for predictive biomarkers—both host and/or tumor features that indicate a high likelihood of response or resistance to a therapy—are urgently needed.

Targeted next generation sequencing DNA assays (tNGS) can identify actionable mutations in DNA from tumors that associate with responses to targeted therapeutics and are used in routine clinical practice in the management of lung cancer, colorectal cancer, and melanoma [2,3]. Although tNGS is not yet in widespread clinical use for prostate cancer, largely due to the paucity of well-defined targets predictive of drug sensitivity, a number of recent studies have indicated potential actionable targets, most notably platinum chemotherapy and poly-ADP ribose polymerase inhibitors (PARPi) for tumors with DNA repair defects [4–8]. Further, an ever-increasing number of clinical trials evaluating targeted agents are underway. UW-OncoPlex is a clinical tNGS panel assay of 194 genes with actionable or potentially actionable mutations [9], and has been extensively used in hematologic malignancies as well as solid tumors such as colorectal, lung, melanoma, sarcoma, and other cancers.

We report our initial experience in a pilot study evaluating UW-OncoPlex in tumors from men undergoing active prostate cancer treatment. The goal of the study was to demonstrate the utility of tNGS testing as applied to prostate cancer patients selected by medical oncologists for unusual clinical and pathologic features, as might occur in real-world clinical practice.

MATERIALS AND METHODS

Patients

Participants were men with prostate cancer receiving care at the genitourinary cancer clinics at the University of Washington and Seattle Cancer Care Alliance (Seattle, WA). Seven medical oncologists identified and recruited men with prostate cancer who were deemed as likely to benefit from tNGS (see additional details below) between the calendar years 2013 and 2015. Forty-five patients signed informed consent to have their tumor material tested using the Clinical Laboratory Improvement Amendments (CLIA)- and College of American Pathologists (CAP)-certified clinical UW-OncoPlex assay. Schema of study flow is shown in Figure 1. Reasons for testing are summarized in Supplementary Table SI.

Ethics Statement

All clinical samples were obtained from subjects who provided written informed consent. The study was performed in accordance with the declaration of Helsinki guidelines and with ethics approval from the Institutional Review Board at the University of Washington.

Target Next-Generation Sequencing Testing

DNA was extracted from fresh or archived FFPE samples, as previously described [9]. H&E-stained slides were reviewed before DNA extraction for all FFPE samples to ensure tumor content, and when feasible, macrodissection of tumor areas was performed to enrich tumor cellularity. UW-OncoPlex is a validated, clinical molecular diagnostic assay that collects simultaneous deep-sequencing information, based on $>500\times$ average coverage, for all classes of mutations in 194 targeted, clinically relevant genes, as previously reported [9]. A clinical report was generated for each tumor sequenced, listing somatic aberrations, potentially actionable findings, along with references from the literature.

Precision Tumor Board

A monthly multidisciplinary precision tumor board was led by a clinical pathologist and attended by medical oncologists, anatomic pathologists, urologists, genetic counselors, research staff, residents, and

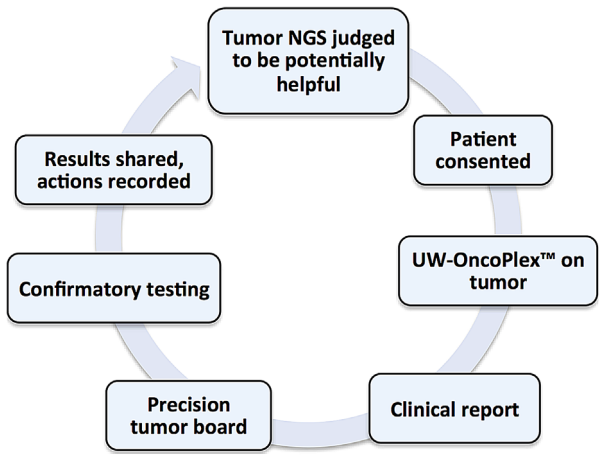


Fig. 1. Schema of pilot study flow.

fellows. Results described in this pilot study were discussed between 4/25/2013 and 2/25/2016. The treating oncologist presented the clinical and family history. The clinical pathologist reviewed and discussed the data analysis and interpretation, and reviewed potentially actionable findings. The group discussed implications for treatment, including clinical trial options, as well as the potential need for referral for genetic counseling. Possible treatments and actions were recorded.

RESULTS

Clinical Features

Of the 45 patients who consented, tumors from 42 patients were sufficient to be evaluated, including 27 primary tumors, 14 metastatic tumors, and 2 salvage surgical specimens. Patient characteristics are summarized in Table I.

In general, UW-OncoPlex was ordered for patients with: (i) an unusual clinical course (i.e., exceptional response to prior therapy); (ii) concern for cancer predisposition (i.e., young age at diagnosis, strong family history of cancers, second primary cancer); and/or (iii) atypical histology. Reasons for testing were varied and listed in detail for each patient in Supplementary Table SI. Testing was done on 11 patients with unusual histologic features, 3 exceptional responders, 23 men diagnosed at $\leq$55 years of age, 12 and 19 men with a family history of prostate or other cancers, respectively, and three with a second primary cancer, with many patients in more than one group.

TABLE I. Clinical Features at Diagnosis and at UW-OncoPlex

Age at diagnosis	
Median (range)–years	57 (45–71)
$\geq$70 years	4 (10%)
$\leq$55 years	20 (48%)
Self-reported race	
White	31 (74%)
African American	7 (17%)
Asian	2 (5%)
Unknown	2 (5%)
Gleason grade sum	
$</=6$	1 (2%)
7	12 (28%)
>7	25 (60%)
Unknown[a]	3 (7%)
Not applicable[b]	1 (3%)
Serum prostate specific antigen at diagnosis	
<4	6 (14%)
4–20	22 (52%)
21–100	7 (17%)
101–1,000	3 (7%)
>1,000	3 (7%)
Unknown	1 (2%)
Localized vs. metastatic at diagnosis	
Localized	29 (69%)
Metastatic	13 (31%)
Disease state at time of UW-OncoPlex	
Localized	14 (33%)
Biochemically recurrent	6 (14%)
Metastatic hormone-sensitive	11 (26%)
Metastatic castration-resistant	9 (21%)
Other metastatic (e.g., squamous cell carcinoma)	2 (5%)

[a]Unknown Gleason score: one received prior ADT, two did not undergo biopsy due to high PSA.
[b]Gleason scoring not applicable due to pure squamous carcinoma histology.

Identification of Somatic Mutations and Clinical Implications

Key findings and actions are shown for the group of patients with non-metastatic and metastatic prostate cancer in Tables II and III, respectively (additional details are available in the corresponding supplementary Tables SII and SIII). Summary integrative analysis is shown in Figure 2. We identified alterations in genes previously reported [4,10], for example: 10/42 (24%), of cases had alterations in genes involved in DNA repair genes (including *BRCA2*, *ATM*, and *CDK12*) similar to recent published reports [7,8]. These patients could be considered for platinum-based chemotherapy [6] or poly-ADP ribose polymerase inhibitor (PARPi) therapy [5]. We have developed a pilot study for such patients using docetaxel/carboplatin for patients with *BRCA1/2* defects (NCT02598895). Another clinical trial option is the pilot study of the PARPi BMN 673 for advanced solid tumors and deleterious BRCA mutations (NCT01989546, available at other institutions) that would not have otherwise been considered.

Other findings with treatment implications included suspected resistance mutations to androgen-receptor pathway inhibitors (such as *AR* amplification, *AR* p.T878A and *AR* p.L702H) observed following treatment with AR-directed agents such as abiraterone and enzalutamide [11,12]. Interestingly, patient 31 was found to have *AR* p.T878A following treatment with just leuprolide and bicalutamide. The presence of this alteration suggests his prostate cancer may not respond to future AR-directed therapies.

Patients with PI3K pathway alterations (including *PIK3R1* insertion, *PIK3R1* mutations, *PIK3CB* mutation and copy gain, and *PIK3CA* mutations) were considered for PI3K-AKT-mTOR inhibitor therapy, including the Phase I trial of AZD8186 (NCT01884285), which was open at our institution. Some cohorts of this study

TABLE II. Key Findings and Resulting Discussion and Actions (Non-Metastatic)

Pt	Key findings	Treatment considerations	Actual therapy change due to UW-OncoPlex	Future therapy	Genetic counseling
		Localized			
26	TMPRSS2-ERG, CHEK2 c.1100delC	Genetic counseling, PARPi, CHEK2 mutation associated with platinum response in ovarian cancer		X	X
38	ATM p.W2960X and ATM p.E2444K, TMPRSS2-ERG	Genetic counseling, consider PARPi and/or platinum		X	X
5	TMPRSS2-ERG	PARPI (TMPRSS2-ERG),		X	
7	MET p.R1327H (R1345H), PTEN p.C296X, TMPRSS2-ERG, TP53 p.G108S with LOH	MET inhibitor (cabozantinib), PI3K-AKT-mTOR inhibitor (PTEN mutation) NCT01884285		X	
13	TMPRSS2-ERG, IKZF1 p.E35K, ABL2 c.347-1G>T	Elected for adjuvant radiation therapy, PARPI (TMPRSS2-ERG)		X	
14	TMPRSS2-ERG	Elected against adjuvant radiation; consider PARPI (TMPRSS2-ERG),		X	
19	KDM6A del exon 17, MEN1 p.V190A, FGFR1 copy loss, AURKA p.V310L, FOXA1 variant	Consider HDAC chromatin remodeling agents (KDM6A), AURKA inhibitor		X	
25	Hypermutation, MSH6 c.1900_1901del with LOH, PTEN c.917_918del, PTEN c.968dup, PTEN c.302T>C, dozens of additional mutations in the context of hypermutation	Consider immune checkpoint inhibitors (Le, et al NEJM, PMID: 26028255); Pembrolizumab for MSI-high, NCT02628067		X	
30	TMPRSS2-ERG, PTEN c.635-1G>C	PARPi (TMPRSS2-ERG)		X	
33	CHD1 p.K1352Vfs*18				
16	(Limited due to low tumor purity) NF1 p. S1380C				
39	Indeterminate				
		Biochemical recurrence			
20	FOXA1 p.N252_G257delinsR, TP53 p.R273H, MYC and FGFR1 copy number gains, TSC1 p.R811Q	Genetic counseling to determine if TP53 p.R273H is germline, mTOR inhibitor (TSC1 variant)		X	X
40	PIK3CA p.A1006V	Consider PI3K-AKT-mTOR inhibitors		X	
23	No actionable findings				
10	(Limited due to poor quality DNA) CDH1 p.R281W				
18	(Limited due to low tumor purity) TP53 p. Y205D, SHH copy gain, FGFR3 copy gain, NOTCH1 copy gain, FOXA1 copy gain				
4	(Limited due to low sequence depth coverage) no actionable findings				

Future therapy defined as current treatment options and/or targeted clinical trials open, pending or expected. Gray text denotes limited study due to low tumor content or poor DNA quality.

required demonstration that the PI3K pathway was altered so the UW-OncoPlex findings both directed therapy and provided clinically useful information for study eligibility.

We also observed RAF fusions (such as *BRAF)*, and mutations in chromatin modifiers (such as *CHD1* and *KDM6A*) that could have implications for therapeutic options, such as enrollment in basket or umbrella trials that are based on specific targets, for example, NCI-Molecular Analysis for Therapy Choice (NCI-MATCH; NCT02465060).

Identification of Suspected Germline Findings

Although UW-OncoPlex is designed to identify somatic mutations, we found a number of gene

TABLE III. Key Findings and Resulting Discussion and Actions (Metastatic)

Pt	Key findings	Treatment considerations	Actual therapy change due to UW-OncoPlex	Future therapy	Genetic counseling
		Metastatic hormone sensitive			
6	ATM p.K2756X and LOH	Based on ATM mutations, genetic counseling, consider future platinum (NCT02598895), consider future PARPi	Enrolled in NCT01576172 (abiraterone +/− veliparib)	X	X
22	FOXA1 p.F254_G257del, AR amplification, RB1 copy loss (likely biallelic loss), APC copy loss (likely biallelic loss), BRAF copy gain, JAK3 p.R175X (possible germline)	Based on JAK3 mutation, genetic counseling. Based on RB1 loss, consider chemotherapy sooner because tumor may behave like AR-null		X	X
34	BRCA2 c.1929delG with LOH, TMPRSS2-ERG, TP53 p.G245C	Based on BRCA2 mutations, genetic counseling, continue future platinum (NCT02598895), consider future PARPi		X	X
1	TMPRSS2-ERG, PTEN copy loss, PIK3R1 in-frame insertion	Based on PTEN loss and PIK3R1 mutation, consider PI3K-AKT-mTOR inhibitors (NCT01884285)		X	
2	TMPRSS2-ERG, TP53 p.G245S	Based on TP53 mutation, consider future Wee1 inhibitor trials		X	
3	BRAF p.K601E, FOXA1 p.F224L, TP53 p.G105_G108del	Based on BRAF mutation, consider BRAF inhibitor or MEK inhibitors		X	
12	TMPRSS2-ERG, CHD1 deletion, RB1 p.R251X with LOH	Based on RB1 loss, consider chemotherapy sooner because tumor may behave like AR-null.		X	
24	TP53 p.R273C, TMPRSS2-ERG, copy number loss chr 2 (including MSH2), chr 6, chr 9; copy gains on portions of chr 1,2,6,17	Based on TP53 mutation, consider future Wee1 inhibitor trials		X	
27	SPOP p.W131R, biallelic inactivation of APC p.R1450X with LOH due to copy loss, CDKN2A homozygous copy loss	CDKN2A loss observed in exceptional responses to taxanes; more confidence in early docetaxel (was already under consideration)		X	
36	PTEN copy loss, PIK3R1 p.W597*, TMPRSS2-ERG	Based on PTEN loss and PIK3R1 mutation, consider PI3K-AKT-mTOR inhibitors (NCT01884285)		X	
		Squamous histology			
28	PIK3R1 m582fs*3 may be gain of function, TP53 p.N29Tfs*15, MYC copy gain, TMPRSS2-ERG fusion and RB exon 4-27 loss and rearrangement	May be more responsive to chemotherapy due to similarities with AR-null tumors	Treated with docetaxel/ carboplatin over AR-directed therapy	X	
8	TP53 c.11101-2A>G, PTEN copy loss, FGFR1 copy gain, NTRK1 p.H772R	Based on suspected germline TP53 mutation, genetic counseling. Based on PTEN loss, consider PI3K-AKT-mTOR inhibitors (NCT01884285). Based on NTRK1 mutation, consider NTRK1 inhibitor trial (NCT02097810). Based on TP53 mutation, consider future Wee1 inhibitor trials		X	X

(Continued)

TABLE III. Continued.

Pt	Key findings	Treatment considerations	Actual therapy change due to UW-OncoPlex	Future therapy	Genetic counseling
		Metastatic castration resistant prostate cancer			
31	AR p.T878A, biallelic inactivation CDK12 c.2495delC AND p.C924G, FOXA1 p.R261C, ABL1 p.F1066L, p. C1067R	Avoid abiraterone (AR p.T878A). Based on CDK12 mutations, consider future platinum (NCT02598895) or PARPi	Avoided abiraterone due to resistance mutation	X	
35	AR p.T878A, PTEN copy loss, MRE11A p.L56W AND p.K633Tfs*45	Based on MRE11A mutations, treat with DNA damaging agent (radium-223), consider future platinum (NCT02598895) or PARPi	Treated with radium-223	X	
11	BRCA2 p.Q3066X, BRCA2 127bp del in exon 11, CHEK2 1100delC, TSC2 c.3883+6G>A	Based on BRCA2 mutations, continue platinum, consider future PARPi. Based on suspected germline BRCA2 mutation, refer to genetic counseling		X	X
17	BRCA2 p.V2969Cfs*7, BRCA2 somatic frame shift deletion, TP53 p.R175H, MLLp.E4650*, AR amplification, APC copy loss	Based on BRCA2 mutations, consider future platinum (NCT02598895) or PARPi. Based on suspected germline BRCA2 mutation, refer to genetic counseling		X	X
15	BRCA2 p.Q2530X and LOH, FOXA1 p. H247R, PTEN copy loss,	Based on BRCA2 mutations, consider future platinum (NCT02598895) or PARPi. Based on suspected germline BRCA2 mutation, refer to genetic counseling. Based on PTEN loss, consider PI3K-AKT-mTOR inhibitor (NCT01884285)		X	X
9	BRCA2 bi-allelic loss, MAP2K1 (MEK1) in-frame del, AR p.W742C, FLT3 p. R387Q, MYC high copy gain	Based on BRCA2 loss, consider future platinum (NCT02598895) or PARPi. May be resistant to anti-androgens.		X	
21	AR amplification, TMPRSS2-ERG fusion, PTEN biallelic inactivation due to large focal deletion	Implications for anti-AR inhibitor resistance. Based on PTEN loss, consider PI3K-AKT-mTOR inhibitors (NCT01884285)		X	
32	PIK3CB p. E1051K mutation, PIK3CB copy gain, PTEN copy loss	Based on PIK3CB mutations, consider PI3K-AKT-mTOR inhibitors (NCT01884285)		X	
29	AR p.L702H, AR high copy gain, SPOP p.F133L, CHD1 homozygous copy loss, APC homozygous copy loss	Consistent with resistance to AR-directed therapies		X	
42	CDK12 c.2963+1G>T and p. H194Kfs*133	Based on CDK12 mutations, consider future platinum (NCT02598895) or PARPi		X	
41	AR partial tandem duplication including exon 3, PTEN homozygous copy loss, BRCA2 copy loss, RB1 copy loss, TP53 mutations	Based on PTEN loss, consider PI3K-AKT-mTOR inhibitors (NCT01884285)		X	
37	MED12 p.L1224F, SF3B1 p.D781G				

ADT, androgen deprivation therapy; TURP, transurethral resection of prostate.
Future therapy defined as current treatment or clinical trial options that were not pursued and/or targeted clinical trials that were pending or anticipated.

Fig. 2. Integrative analysis of genetic aberrations identified through targeted next generation sequencing. Columns represent individual affected individuals, and rows represent specific genes grouped in pathways. Color legend of the aberrations represented including amplification, gain, deep deletion, shallow deletion, missense, inframe/frameshift, truncated, and intronic. Cased with more aberrations in a gene are represented by split colors. Confirmed germline mutations are indicated with a gray box.

alterations that were suspected to be germline mutations in high-penetrance cancer risk genes, such as in *BRCA2* in 4/42 (10%) cases and *TP53* in 2/42 (5%) cases. We also found mutations in moderate-penetrance cancer risk genes, such as *CHEK2* 2/42 (5%) [13]. Other mutations were found in genes conferring risk for other cancers such as breast and ovarian, but are not yet well established as prostate cancer risk genes, such as *ATM* in 2/42 (5%). Overall, 10/42 (24%) had suspected deleterious germline mutations genes with potential implications for inherited cancer predisposition. These patients were informed that the tumor sequencing results suggested the possibility of an inherited cancer risk gene and were referred for genetic evaluation, of which seven patients were confirmed. In two cases, results were not available until after death, but families were notified of the findings and offered referral to genetic counseling.

DISCUSSION

The molecular characterization of cancers using tNGS assays has the potential to enhance the outcomes of cancer patients by efficiently identifying patients with specific tumor features that confer drug susceptibility and/or drug resistance, resulting in personalized cancer treatment strategies. In prostate cancer there is not yet routine use of tNGS testing, in part because targets linked to treatment options have not yet been as well-defined and validated as in colorectal cancer, lung cancer, and melanoma [2,3]. The past several years have brought not only better understanding of the molecular profiles of prostate cancer, but also increasing availability and ease of access to tNGS testing platforms, including and FoundationOne, even to community providers. We conducted this pilot study to assess the feasibility and utility of integrating a tNGS in the setting of a real-world prostate cancer clinical practice using the UW-OncoPlex platform.

One observation that became apparent in the precision tumor board discussions is a lack of standard criteria for defining clinical actionability, which varies widely depending on the stringency of criteria used and including the level of evidence and the disease context. Here, we found it useful to delineate three categories of action at the time of results reporting (shown in Tables II and III): First, actual therapy change, rigorously defined as a direct change in treatment that would not have occurred were it not for the UW-OncoPlex results. Second, future therapy implications, which we defined as treatment options or clinical trials that were suggested by UW-OncoPlex results, but were not immediately chosen and/or were pending or anticipated. For example, patients whose results pointed toward a target for which a clinical trial of a targeted agent existed but was not yet open at our institution, or patients with localized disease or biochemical recurrence with no need for immediate treatment, but who might need treatment options down the line. Third, immediate genetic counseling implications, defined as results from tumor testing suggesting possible germline mutation in a cancer predisposition gene (irrespective of family history of cancer).

We found a high proportion of findings that were suspected to be germline based on tumor-only testing, not entirely surprising given that a majority (but not all) had a positive family history of cancer. In such cases, referral for genetic counseling was recommended. Several groups including American Society of Clinical Oncology and National Cancer Institute have recently published thoughtful discussions and guiding recommendations regarding germline implications of tumor testing [14,15]. A report describing NGS testing of 1566 solid tumors tested found that 13% had mutations in genes associated with inherited cancer susceptibility [16]. A study of 1,915 ovarian cancers determined that 18% carried germline mutations associated with ovarian cancer risk [17]. For prostate cancer, as with other tumors that undergo somatic profiling, managing the information and its implications is an important consideration for ordering providers. Secondary findings such as suspected

germline mutations come with complex decision-making dilemmas, but also offer another avenue for significant potential medical benefit to patients and their families.

Our pilot study was initiated with the goal of evaluating the feasibility and utility of integrating NGS testing into clinical practice in our dedicated prostate cancer clinics. We intentionally allowed flexibility of patient selection criteria so that medical oncologists could have the freedom to explore what tNGS could offer in terms of illuminating treatment options. Because oncologists selected patients considered "high risk" for significant somatic and germline genetic alterations, it was not surprising that we found a relatively high frequency of mutations associated with more aggressive disease and germline mutations. For example, this pilot cohort has a high rate of actionable findings as well as a higher estimated rate of potential germline findings compared to recent estimates [7] (Pritchard, et al. NEJM, in press).

Although UW-OncoPlex testing in the localized or biochemical relapse settings yielded numerous findings, we observed that there were no clinical trials of targeted agents that were available and appropriate for our patients in the non-metastatic setting. However, this is likely to change over time as the recent observation that DNA repair mutations may predict response to platinum chemotherapy and PARP inhibition may lead to trials of these agents in the earlier disease settings in selected patients whose cancers bear these mutations.

Limitations to our study are the small numbers inherent to a pilot study. We did not set strict entry criteria, but rather allowed treating oncologists to determine when they felt testing might be useful. As a consequence, the patient population is heterogeneous. In addition, we do not have extensive follow-up data to demonstrate that tNGS-directed therapy leads to better outcomes compared to empiric therapy choices. Future studies will be needed to further investigate these measures.

In summary, this pilot study suggests a role for tNGS of prostate cancer in guiding therapy planning including treatment and clinical trial selection. In addition, the possibility that tumor sequencing may suggest and indicate the presence of inherited mutations in cancer predisposition genes is important to counsel and prepare patients for, and may have far-reaching implications for genetic counseling, screening, and prevention in family members. Medical providers caring for prostate cancer patients should be alert to and prepared for the possibility that tumor testing may uncover potential germline findings with implications for genetic counseling.

CONCLUSIONS

Targeted next generation sequencing of prostate cancers in the context of a prostate cancer precision tumor board is feasible, and has the potential to guide and prioritize timely discussions around the identification of consequential variants.

ACKNOWLEDGMENTS

We are especially grateful to the patients who generously volunteered to participate in this study. In addition, we thank: Bob Livingston, University of Washington; and Hiep Nguyen University of Washington, for their help with this study.

REFERENCES

1. Basch E, Loblaw DA, Oliver TK, Carducci M, Chen RC, Frame JN, Garrels K, Hotte S, Kattan MW, Raghavan D, Saad F, Taplin ME, Walker-Dilks C, Williams J, Winquist E, Bennett CL, Wootton T, Rumble RB, Dusetzina SB, Virgo KS. Systemic therapy in men with metastatic castration-resistant prostate cancer: American Society of Clinical Oncology and Cancer Care Ontario clinical practice guideline. J Clin Oncol 2014; 32(30):3436–3448.
2. Hagemann IS, Devarakonda S, Lockwood CM, Spencer DH, Guebert K, Bredemeyer AJ, Al-Kateb H, Nguyen TT, Duncavage EJ, Cottrell CE, Kulkarni S, Nagarajan R, Seibert K, Baggstrom M, Waqar SN, Pfeifer JD, Morgensztern D, Govindan R. Clinical next-generation sequencing in patients with non-small cell lung cancer. Cancer 2015;121(4):631–639.
3. Fisher KE, Zhang L, Wang J, Smith GH, Newman S, Schneider TM, Pillai RN, Kudchadkar RR, Owonikoko TK, Ramalingam SS, Lawson DH, Delman KA, El-Rayes BF, Wilson MM, Sullivan HC, Morrison AS, Balci S, Adsay NV, Gal AA, Sica GL, Saxe DF, Mann KP, Hill CE, Khuri FR, Rossi MR. Clinical validation and implementation of a targeted next-generation sequencing assay to detect somatic variants in non-small cell lung, melanoma, and gastrointestinal malignancies. J Mol Diagn 2016;18(2):299–315.
4. Beltran H, Yelensky R, Frampton GM, Park K, Downing SR, MacDonald TY, Jarosz M, Lipson D, Tagawa ST, Nanus DM, Stephens PJ, Mosquera JM, Cronin MT, Rubin MA. Targeted next-generation sequencing of advanced prostate cancer identifies potential therapeutic targets and disease heterogeneity. Eur Urol 2013;63(5):920–926.
5. Mateo J, Carreira S, Sandhu S, Miranda S, Mossop H, Perez-Lopez R, Nava Rodrigues D, Robinson D, Omlin A, Tunariu N, Boysen G, Porta N, Flohr P, Gillman A, Figueiredo I, Paulding C, Seed G, Jain S, Ralph C, Protheroe A, Hussain S, Jones R, Elliott T, McGovern U, Bianchini D, Goodall J, Zafeiriou Z, Williamson CT, Ferraldeschi R, Riisnaes R, Ebbs B, Fowler G, Roda D, Yuan W, Wu YM, Cao X, Brough R, Pemberton H, A'Hern R, Swain A, Kunju LP, Eeles R, Attard G, Lord CJ, Ashworth A, Rubin MA, Knudsen KE, Feng FY, Chinnaiyan AM, Hall E, de Bono JS. DNA-repair defects and olaparib in metastatic prostate cancer. N Engl J Med 2015;373(18): 1697–1708.
6. Cheng HH, Pritchard CC, Boyd T, Nelson PS, Montgomery B. Biallelic inactivation of BRCA2 in pplatinum-sensitive metastatic castration-resistant prostate cancer. Eur Urol 2016; 69(6):996–997.

7. Robinson D, Van Allen EM, Wu YM, Schultz N, Lonigro RJ, Mosquera JM, Montgomery B, Taplin ME, Pritchard CC, Attard G, Beltran H, Abida W, Bradley RK, Vinson J, Cao X, Vats P, Kunju LP, Hussain M, Feng FY, Tomlins SA, Cooney KA, Smith DC, Brennan C, Siddiqui J, Mehra R, Chen Y, Rathkopf DE, Morris MJ, Solomon SB, Durack JC, Reuter VE, Gopalan A, Gao J, Loda M, Lis RT, Bowden M, Balk SP, Gaviola G, Sougnez C, Gupta M, Yu EY, Mostaghel EA, Cheng HH, Mulcahy H, True LD, Plymate SR, Dvinge H, Ferraldeschi R, Flohr P, Miranda S, Zafeiriou Z, Tunariu N, Mateo J, Perez-Lopez R, Demichelis F, Robinson BD, Schiffman M, Nanus DM, Tagawa ST, Sigaras A, Eng KW, Elemento O, Sboner A, Heath EI, Scher HI, Pienta KJ, Kantoff P, de Bono JS, Rubin MA, Nelson PS, Garraway LA, Sawyers CL, Chinnaiyan AM. Integrative clinical genomics of advanced prostate cancer. Cell 2015;161(5):1215–1228.
8. Cancer Genome Atlas Research. The molecular taxonomy of primary prostate cancer. Cell 2015;163(4):1011–1025.
9. Pritchard CC, Salipante SJ, Koehler K, Smith C, Scroggins S, Wood B, Wu D, Lee MK, Dintzis S, Adey A, Liu Y, Eaton KD, Martins R, Stricker K, Margolin KA, Hoffman N, Churpek JE, Tait JF, King MC, Walsh T. Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. J Mol Diagn 2014;16(1):56–67.
10. Grasso CS, Wu YM, Robinson DR, Cao X, Dhanasekaran SM, Khan AP, Quist MJ, Jing X, Lonigro RJ, Brenner JC, Asangani IA, Ateeq B, Chun SY, Siddiqui J, Sam L, Anstett M, Mehra R, Prensner JR, Palanisamy N, Ryslik GA, Vandin F, Raphael BJ, Kunju LP, Rhodes DR, Pienta KJ, Chinnaiyan AM, Tomlins SA. The mutational landscape of lethal castration-resistant prostate cancer. Nature 2012;487(7406):239–243.
11. Chen EJ, Sowalsky AG, Gao S, Cai C, Voznesensky O, Schaefer R, Loda M, True LD, Ye H, Troncoso P, Lis RL, Kantoff PW, Montgomery RB, Nelson PS, Bubley GJ, Balk SP, Taplin ME. Abiraterone treatment in castration-resistant prostate cancer selects for progesterone responsive mutant androgen receptors. Clin Cancer Res 2015;21(6):1273–1280.
12. Romanel A, Gasi Tandefelt D, Conteduca V, Jayaram A, Casiraghi N, Wetterskog D, Salvi S, Amadori D, Zafeiriou Z, Rescigno P, Bianchini D, Gurioli G, Casadio V, Carreira S, Goodall J, Wingate A, Ferraldeschi R, Tunariu N, Flohr P, De Giorgi U, de Bono JS, Demichelis F, Attard G. Plasma AR and abiraterone-resistant prostate cancer. Sci Transl Med 2015; 7(312):312re10.
13. Naslund-Koch C, Nordestgaard BG, Bojesen SE. Increased risk for other cancers in addition to breast cancer for CHEK2*1100delC heterozygotes estimated from the Copenhagen General Population Study. J Clin Oncol 2016;34(11): 1208–1216.
14. Raymond VM, Gray SW, Roychowdhury S, Joffe S, Chinnaiyan AM, Parsons DW, Plon SE, G. Clinical Sequencing Exploratory Research Consortium Tumor Working. Germline findings in tumor-only sequencing: Points to consider for clinicians and laboratories. J Natl Cancer Inst 2016;108(4): djv351.
15. Robson ME, Bradbury AR, Arun B, Domchek SM, Ford JM, Hampel HL, Lipkin SM, Syngal S, Wollins DS, Lindor NM. American Society of Clinical Oncology Policy Statement Update: Genetic and genomic testing for cancer susceptibility. J Clin Oncol 2015;33(31):3660–3667.
16. Schrader KA, Cheng DT, Joseph V, Prasad M, Walsh M, Zehir A, Ni A, Thomas T, Benayed R, Ashraf A, Lincoln A, Arcila M, Stadler Z, Solit D, Hyman D, Zhang L, Klimstra D, Ladanyi M, Offit K, Berger M, Robson M. Germline variants in targeted tumor sequencing using matched normal DNA. JAMA Oncol 2016;2(1):104–111.
17. Norquist BM, Harrell MI, Brady MF, Walsh T, Lee MK, Gulsuner S, Bernards SS, Casadei S, Yi Q, Burger RA, Chan JK, Davidson SA, Mannel RS, DiSilvestro PA, Lankes HA, Ramirez NC, King MC, Swisher EM, Birrer MJ. Inherited mutations in women with ovarian carcinoma. JAMA Oncol 2016;2(4): 482–490.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

available at www.sciencedirect.com
journal homepage: www.europeanurology.com

European Association of Urology

Platinum Priority – Review – Prostate Cancer

Editorial by XXX on pp. x–y of this issue

DNA Repair in Prostate Cancer: Biology and Clinical Implications

Joaquin Mateo [a,b], *Gunther Boysen* [a], *Christopher E. Barbieri* [c,d,e], *Helen E. Bryant* [f], *Elena Castro* [g], *Pete S. Nelson* [h,i], *David Olmos* [g,j], *Colin C. Pritchard* [h], *Mark A. Rubin* [d,e,k], *Johann S. de Bono* [a,b,*]

[a] *Division of Cancer Therapeutics and Division of Clinical Studies, The Institute of Cancer Research, London, UK;* [b] *Drug Development Unit, The Royal Marsden NHS Foundation Trust, London, UK;* [c] *Department of Urology, Weill Cornell Medicine, New York, NY, USA;* [d] *Caryl and Israel Englander Institute for Precision Medicine, New York Presbyterian Hospital-Weill Cornell Medicine. New York, NY, USA;* [e] *Sandra and Edward Meyer Cancer Center, Weill Cornell Medicine, New York, NY, USA;* [f] *Sheffield Institute for Nucleic Acids, Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK;* [g] *Prostate Cancer Unit, Spanish National Cancer Research Centre, Madrid, Spain;* [h] *Department of Laboratory Medicine, University of Washington, Seattle, WA, USA;* [i] *Divisions of Human Biology and Clinical Research, Fred Hutchinson Cancer Research Center, University of Washington, Seattle, WA, USA;* [j] *Medical Oncology Department, CNIO-IBIMA Genitourinary Cancer Unit, Hospital Virgen de la Victoria and Hospital Regional de Malaga, Malaga, Spain;* [k] *Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY, USA*

Article info

Article history:
Accepted August 12, 2016

Associate Editor:
James Catto

Keywords:
Prostate cancer
DNA repair
PARP
BRCA
Personalized medicine
DNA damage

Abstract

Context: For more precise, personalized care in prostate cancer (PC), a new classification based on molecular features relevant for prognostication and treatment stratification is needed. Genomic aberrations in the DNA damage repair pathway are common in PC, particularly in late-stage disease, and may be relevant for treatment stratification.
Objective: To review current knowledge on the prevalence and clinical significance of aberrations in DNA repair genes in PC, particularly in metastatic disease.
Evidence acquisition: A literature search up to July 2016 was conducted, including clinical trials and preclinical basic research studies. Keywords included *DNA repair, BRCA, ATM, CRPC, prostate cancer, PARP, platinum, predictive biomarkers*, and *hereditary cancer*.
Evidence synthesis: We review how the DNA repair pathway is relevant to prostate carcinogenesis and progression. Data on how this may be relevant to hereditary cancer and genetic counseling are included, as well as data from clinical trials of PARP inhibitors and platinum therapeutics in PC.
Conclusions: Relevant studies have identified genomic defects in DNA repair in PCs in 20–30% of advanced castration-resistant PC cases, a proportion of which are germline aberrations and heritable. Phase 1/2 clinical trial data, and other supporting clinical data, support the development of PARP inhibitors and DNA-damaging agents in this molecularly defined subgroup of PC following success in other cancer types. These studies may be an opportunity to improve patient care with personalized therapeutic strategies.
Patient summary: Key literature on how genomic defects in the DNA damage repair pathway are relevant for prostate cancer biology and clinical management is reviewed. Potential implications for future changes in patient care are discussed.

© 2016 European Association of Urology. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

* Corresponding author. Division of Clinical Studies, The Institute of Cancer Research Drug Development Unit, The Royal Marsden NHS Foundation Trust, Downs Road, Sutton SM2 5PT, UK. Tel. +44 208 7224028; Fax: +44 208 6427979.
E-mail address: johann.de-bono@icr.ac.uk (J.S. de Bono).

http://dx.doi.org/10.1016/j.eururo.2016.08.037
0302-2838/© 2016 European Association of Urology. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Please cite this article in press as: Mateo J, et al. DNA Repair in Prostate Cancer: Biology and Clinical Implications. Eur Urol (2016), http://dx.doi.org/10.1016/j.eururo.2016.08.037

1. Introduction

While therapeutic options for patients with advanced prostate cancer (PC) have improved over the last decade, castration-resistant PC (CRPC) remains a lethal disease [1]. Recently, relevant studies have identified genomic defects in DNA repair in advanced and primary PC. This has led to clinical studies that provide a strong rationale for developing PARP inhibitors and DNA-damaging agents in this molecularly defined PC subgroup. Following the successful development of targeted agents for molecularly defined subpopulations in other cancer types [2,3], there may be an opportunity to potentially improve patient care in PC via personalized therapeutic strategies. In this article, we review the biology and clinical implications of deleterious inherited or acquired DNA repair pathway aberrations in PC.

2. Evidence acquisition

A literature search for clinical trials and preclinical basic research studies up to July 2016 was conducted. Keywords for the included "DNA repair", "BRCA", "ATM", "CRPC", "prostate cancer", "PARP", "platinum", "predictive biomarkers", and "hereditary cancer".

3. Evidence synthesis

3.1. The molecular landscape of primary and advanced PC

Advances in genomics have permitted the identification of putative drivers of carcinogenesis and cancer progression. These genomic data provide for precise molecular tumor subclassification that extends beyond traditional histologic descriptions. For optimal utility, molecular clusters should provide prognostic or predictive information relevant for patient care [4].

Several studies have depicted the genomic landscape of primary prostate tumors [5–7]. Recently, The Cancer Genome Atlas Research Network (TCGA) reported on whole-exome sequencing of a series of 333 localized PCs [8]. Seven subgroups were defined on the basis of certain gene fusions involving the ERG/ETS transcription factor family (ERG, ETV1/4, and FLI1) or recurrent mutations in specific genes (*SPOP*, *FOXA1*, and *IDH1*); these subgroups differ with regard to androgen receptor (AR) signaling activity, DNA methylation, and microRNA expression.

In the TCGA study, in which Gleason ≥8 tumors represented 26% of the cohort, 62/333 (19%) tumors had deleterious germline or somatic aberrations in genes key to the DNA damage repair pathway (*BRCA2*, *BRCA1*, *CDK12*, *ATM*, *FANCD2*, *RAD51C*). Six of these aberrations involved a *BRCA2* K3326* nonsense germline variant, which arguably does not greatly impact protein function despite a modest association with risk of cancer [9], and 23 cases had heterozygous deletions of *FANCD2* or *RAD51* without evidence of biallelic inactivation; consequently, the proportion of localized PCs with impaired DNA repair function is probably less than 19%.

Next-generation sequencing studies of metastatic tumors identified enrichment of mutations in DNA repair genes among patients with lethal disease [10,11]. To provide a systematic analysis of the genomic landscape of CRPC and its potential relevance for patient care, the Stand Up To Cancer (SU2C)-Prostate Cancer Foundation (PCF) International Dream Team pursued whole-exome and transcriptome sequencing of 150 biopsies from metastatic CRPC (mCRPC) [12]. Higher prevalence of aberrations in key DNA repair genes (23%), *TP53* (53%), *RB1* (21%), the PTEN-PI3K pathway (49%), and *AR* (63%) in mCRPC than in localized disease was confirmed. It is not yet clear if this enrichment is secondary to a tumor evolution process in response to therapy exposure, or purely suggests markers of more aggressive PCs (Table 1).

Table 1 – Prevalence of DNA repair gene mutations and deletions described in studies on localized and metastatic prostate cancer

Study	Disease status	Samples	Gene frequency							
		(*n*)	Homologous recombination				MMR		NER	
SU2C-PCF CRPC genomic landscape [12]	CRPC metastasis	150	*BRCA1*	0.7%	*CDK12*	4.7%	*MLH1*	1.3%	*ERCC2*	1.3%
			BRCA2	13.3%	*CHEK2*	3.0%	*MSH2*	3.0%	*ERCC5*	1.3%
			ATM	7.3%	*PALB2*	2.0%	*MSH6*	2.0%		
UM PC genomics [11]	CRPC metastasis	50	*BRCA1*	0%	*CDK12*	6.0%	*MLH1*	2.0%	*ERCC2*	2.0%
			BRCA2	12.0%	*CHEK2*		*MSH2*	2.0%	*ERCC5*	12.0%
			ATM	6.0%	*PALB2*	0%	*MSH6*	2.0%		
UM PC genomics [11]	Treatment-naïve tumors	11	*BRCA1*	0%	*CDK12*	0	*MLH1*	0	*ERCC2*	0
			BRCA2	1/11	*CHEK2*	0	*MSH2*	1/11	*ERCC5*	0
			ATM	1/11	*PALB2*	0	*MSH6*	1/11		
Weill Cornell/Broad [6]	Prostatectomy for localized or locally advanced PC (somatic only)	109	*BRCA1*	1.8%	*CDK12*	0	*MLH1*	0	*ERCC2*	0
			BRCA2	0%	*CHEK2*	0	*MSH2*	0	*ERCC5*	0
			ATM	2.8%	*PALB2*	1.8%	*MSH6*	0.9%		
TCGA localized PC [8]	Localized PC	333	*BRCA1*	1.0%	*CDK12*	2.0%	*MLH1*	0.3%	*ERCC2*	0.6%
			BRCA2	3.0%*	*CHEK2*	0%	*MSH2*	0.3%	*ERCC5*	0.3%
			ATM	4.0%	*PALB2*	0%	*MSH6*	1.5%		

MMR = mismatch repair; NER = nucleotide-excision repair; PC = prostate cancer; CRPC = castration-resistant PC; SU2C-PCF = Stand Up To Cancer-Prostate Cancer Foundation; UM = University of Michigan; TCGA = The Cancer Genome Atlas.

Please cite this article in press as: Mateo J, et al. DNA Repair in Prostate Cancer: Biology and Clinical Implications. Eur Urol (2016), http://dx.doi.org/10.1016/j.eururo.2016.08.037

With regard to DNA repair genes, the SU2C-PCF study identified inactivation of key DNA repair genes in at least 23% of cases, including homologous recombination (HR)–mediated repair genes (most commonly *BRCA2* and *ATM*) and mismatch repair (MMR) genes (*MLH1*, *MSH2*). Other DNA repair mechanisms are also likely to be impacted because of known influences of the AR in nonhomologous end-joining (NHEJ), and possibly aberrations in nucleotide excision repair (NER) and base excision repair (BER).

Intrapatient tumor heterogeneity represents a challenge for genomic stratification of PC in the clinic. Several studies have comprehensively observed an overall higher degree of heterogeneity within primary prostate tumors than in advanced disease [13–15]. This is likely to be related to: (1) bottlenecks in the metastatic process that limit metastatic spread and growth; (2) the capacity of metastatic tumor cells to seed other metastasis and even reseed the primary tumor; and (3) the selection of resistant clones driven by treatment exposures. Alterations in DNA repair genes have been related to increased mutational burden and may generate increased intrapatient heterogeneity; specific studies addressing the impact of genomic instability on treating the diverse subtypes of this common disease are now needed.

3.2. The DNA damage response pathway: a general overview

At any time, the DNA in human cells is constantly being damaged. If there is a deficient repair of this damage, genome stability is compromised, which can contribute to tumorigenesis. Damage can occur endogenously (due to spontaneous hydrolysis of bases or reaction of DNA with naturally occurring reactive oxygen species or alkylating agents) or can be induced by exogenous agents (eg, radiation and toxins). To protect their genome integrity, cells have evolved a complex signaling machinery for recognizing and repairing damage that includes several pathways with complementary and partially overlapping functions. Different forms of DNA damage trigger a response from different branches of this complex system. The main workflow is as follows; when genomic insults are detected, cell-cycle checkpoints are activated to halt the cell cycle and allow the cellular machinery to repair the DNA damage. If the repair is successful, the cell can continue its normal cycle; otherwise, programmed cell death or senescence programs are triggered. If the DNA repair mechanisms are dysfunctional, genomic instability, which is one of the hallmarks of carcinogenesis, ensues.

When damage is limited to one of the DNA strands (single-strand breaks or base modifications), different repair mechanisms can be deployed. These include BER, single-strand break repair (SSBR), NER, and MMR. Each of these pathways uses the complementary undamaged strand as a template to ensure fidelity of repair. BER is mainly activated to repair endogenous oxidative or alkylated base damage [16]. PARP1 and PARP2 are involved in detecting single-strand breaks, which are formed either directly or as intermediates in BER, and help to coordinate the SSBR response. The NER machinery is responsible for repairing bulky adducts such as those induced by UV light, for which the ERCC family of proteins are key mediators. The MMR pathway corrects mutations formed during DNA replication and recombination. The MSH and MLH family of genes are, among others, critical for MMR. The primary mechanisms involved in DNA double-strand break (DSB) repair comprise the HR system and NHEJ. HR requires a sister chromatid as template and is therefore restricted to the S/G2 phases of the cell cycle. It restores the original DNA code error-free. Key mediators of this pathway include BRCA1, BRCA2, PALB2, ATM, ATR, RAD51, MRE11, CHEK2, and XRCC2/3. In contrast, NHEJ functions by ligating broken DNA ends without the use of a template and is therefore functional throughout the cell cycle. The error-prone mode of NHEJ action leads to errors that are permanent and can drive genomic instability (Fig. 1). SSBs that are not repaired before DNA replication takes place will collapse replication forks, leading to formation of DSBs, which then require HR for repair and continued replication [17].

3.3. DNA repair defects play a relevant role in carcinogenesis and PC progression

Prostate carcinogenesis is mediated, as in other cancers, by the accumulation of genetic and epigenetic aberrations; these molecular changes can be inherited or be the result of altered AR transcriptional activity, changes in chromatin architecture, oncogenic replication, error-prone DNA repair, or defective cell division. The sum of these processes confers survival and growth advantage to the transformed cell. Many of these alterations are induced by factors of the microenvironment, particularly the immune system. Chronic inflammation with continued oxidative stress contributes to carcinogenesis of the prostate epithelium by inducing genomic damage. Deficient DNA repair response and defective apoptotic checkpoint control can then lead to permanent incorporation of these genome abnormalities.

AR signaling is critical not only for normal development of the prostate gland but also for prostate carcinogenesis. Genomic instability is related to AR transcriptional activity, and the cross-regulation between AR signaling and DNA damage response pathways appears to be relevant for PC progression [18]. Nevertheless, the role of AR in genome instability is only partly understood [19,20].

Rearrangements between the androgen-regulated *TMPRSS2* gene and the ETS genes *ERG*, *ETV1*, and *ETV4* are common in PC; these appear to be early events contributing to, but not sufficient on their own, prostate carcinogenesis, and are at least partly lineage-specific [5]. AR-driven transcription can result in increased DNA DSB generation at transcriptional hubs, probably as a result of topoisomerase-IIβ enzyme activity, leading to complex structural rearrangements across the genome [21,22]. Mechanistically, this is supported by AR binding to specific chromosomal sites creating a proximity to otherwise distant chromatin loci [20]. *TMPRSS2-ERG* translocation is probably the commonest example of such processes [23,24].

Interestingly, some PCs are characterized by high numbers of rearrangements. Many of these tumors have

Please cite this article in press as: Mateo J, et al. DNA Repair in Prostate Cancer: Biology and Clinical Implications. Eur Urol (2016), http://dx.doi.org/10.1016/j.eururo.2016.08.037

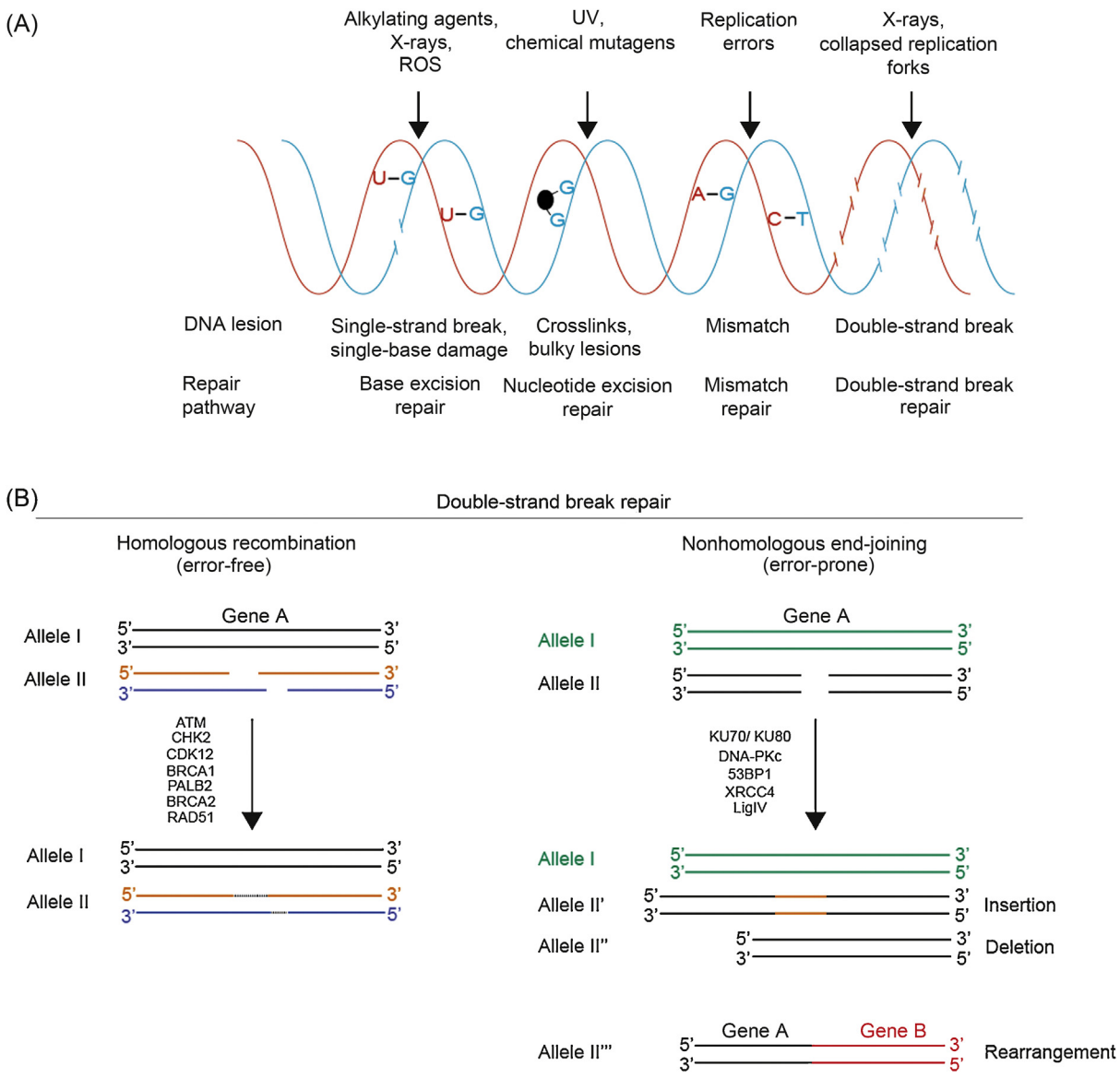


Fig. 1 – An overview of the most important DNA damage–inducing stresses and the corresponding molecular pathways that eukaryotic cells established to repair these. Diverse environmental and endogenous stresses can damage DNA, causing either single-strand (lilac) or double-strand (red box) DNA breaks. Eukaryotic cells developed various molecular mechanisms that repair such damage. DNA double-strand breaks are the most toxic DNA damage, and can be lethal for a cell if not properly repaired. The two most common and best-studied DNA double-strand repair pathways are homologous recombination (HR; error-free) and nonhomologous end-joining (NHEJ; error-prone). HR is limited to the S/G2 phases of the cell cycle and requires a sister chromatid as repair template. Key pathway components are highlighted. NHEJ is active mostly during the G1 phase of the cell cycle and can lead to structural genomic alterations (rearrangements), loss of genomic material (deletion), or insertion of additional nucleotides as a consequence of its imprecise nature. Key pathway components are highlighted.

oncogenic mutations in the *SPOP* gene that stabilize proteins including AR and its transcriptional regulators. Mechanistically, *SPOP* mutant tumors rely predominantly on NHEJ-based DSB repair (while reducing error-free HR-mediated DSB repair activity) [25].

The pattern of genomic aberrations may partly depend on deficiencies in specific DNA repair pathway branches. It has been shown that loss of MMR function induces a hypermutated microsatellite unstable genotype [12]. Somatic complex rearrangements in *MSH2* and *MSH6*, as well as somatic and germline truncating mutations in these two genes, have been described as the most common mechanism for MMR-deficient prostate tumors [26,27]. BRCA2-deficient PCs also present specific mutation signatures enriched in deletions and with higher mutational burden than wild-type–BRCA2 tumors [28,29].

3.4. *Inherited mutations in DNA repair genes and PC risk*

Hereditary germline mutations in DNA repair genes are associated with a higher risk of PC. This results in one gene allele being dysfunctional in every cell, with the second

allele commonly lost by a second hit (mutation, deletion, epigenetic silencing) [30]. Germline mutations in *BRCA2* increase the risk of developing PC (relative risk 8.6 in men <65 yr) [31,32]; their role in the development and progression of breast, ovarian, and pancreatic cancers is also well established. Moreover, inherited mutations in other DNA repair genes such as *PALB2*, *MLH1*, *MSH2*, and *PMS2* also appear to be associated with PC risk [33]. While the proportion of patients carrying a germline *BRCA1/2* mutation is low (1–2%) among the general population of primary PC patients, a multicenter study lead by the SU2C-PCF consortium in metastatic PC patients estimated the prevalence of germline *BRCA2* mutations as 5.3% in the setting of advanced disease; when a panel of 20 DNA repair genes was considered, 82/692 (11.8%) of patients with metastatic disease carried an underlying germline mutation [34]. Interestingly, age at diagnosis and family history of PC did not identify the mutation carriers, although there was enrichment among patients with a family history of cancer. It is therefore now critical to reconsider current guidelines for germline DNA testing; this could be relevant not only for treatment stratification but also in triggering cascade genetic testing for relatives who may be candidates for targeted cancer screening programs.

At present there is no consensus on how to manage this high-risk population with regard to screening for PC. To address this issue, the IMPACT study is evaluating targeted PC screening in men with germline *BRCA1/2* (gBRCA1/2) mutations. Annual prostate-specific antigen (PSA) tests are performed, and a biopsy is triggered if PSA >3ng/ml. A total of 1522 gBRCA1/2 mutation carriers and 959 controls had been recruited at last reporting. Preliminary results have revealed a higher incidence of PC in g*BRCA2* mutation carriers (3.3% vs 2.6% in g*BRCA1* mutation carriers, <2% for controls), who also have a higher likelihood of intermediate/high risk. Final results from these studies are awaited to ascertain the optimal screening strategies for this population [35].

Inherited mutations impairing the MMR function (Lynch syndrome) have been associated with an almost fivefold higher risk of PC, although additional work is needed to determine precise risks [36].

3.5. Impact of DNA repair defects on clinical outcome and response to treatment in PC

The relevance of somatic loss of function of DNA repair genes in the treatment of CRPC is still not clear, as neither the TCGA (primary tumors) nor the SU2C/PCF (metastatic disease) landscape studies reported follow-up clinical outcome data. Prospective studies looking at whether this molecular classification results in clinically relevant stratification for prognosis and treatment response are needed [8,12]. There are data on clinical outcome according to gBRCA1/2 in localized disease. In a series of more than 2000 patients with localized PC, including 61 *BRCA2* and 18 *BRCA1* mutation carriers, 23% of gBRCA1/2 mutation carriers developed metastasis after 5 yr of radical treatment, compared to 7% of noncarriers ($p = 0.001$). Cause-specific survival was significantly shorter among carriers (8.6 yr) compared to noncarriers (15.7 yr; $p = 9 \times 10^{-8}$). Subgroup analysis confirmed g*BRCA2* mutations as independent factor for poor prognosis [37]. The poorer outcome for g*BRCA2* mutation carriers seems to be particularly relevant for patients treated with radical radiotherapy in comparison to surgery, although the patient numbers evaluated were too small to support a robust claim [38]. The exact biological reasons underlying this poorer outcome remain to be fully elucidated; data from small series suggest that these tumors remain sensitive to taxanes [39,40].

3.6. Using DNA repair defects as a therapeutic target: PARP inhibitors

Over the last decade, exploitation of the vulnerabilities of tumor cells with DNA repair gene defects has been pursued in different tumor types, most successfully in ovarian and breast cancers. The identification of a subgroup of mCRPC with DNA repair defects with a similar genomic profile provides a strong rationale for developing the same therapeutic strategies for this molecular subtype of PC [10].

Poly (ADP-ribose) polymerases (PARP) are a family of enzymes involved, primarily, in transcriptional regulation and in detecting and localizing other DNA repair proteins to DNA single strand breaks. Activation of PARP1 and PARP2 triggers the damage response and recruits of key effectors of repair.

The fundamental basis for inhibiting PARP as anticancer therapy is the established biological concept called synthetic lethality: two genomic events that are each relatively innocuous individually become lethal when occurring together [41]. When PARP1/2 are pharmacologically inhibited, SSBs cannot be repaired and eventually progress to toxic DSBs. If a cell is competent in repairing damage, it will be able to fix the DSB. However, if a cell is lacking HR repair capacity (eg, *BRCA1*, *BRCA2*, *PALB2* or *ATM* is dysfunctional or lost), then PARP inhibiton would become lethal.

Two landmark studies demonstrated in 2005 specific killing of cell lines in which BRCA1/2 had been silenced or lost by the PARP inhibitor (PARPi) KU-0059436 (later named AZD2281, olaparib) [42,43]. In these studies, PARP inhibition led to γH2AX accumulation and the absence of RAD51 foci formation in BRCA-deficient models. Subsequent studies have revealed similar effects for other PARP inhibitors now in clinical development, and demonstrated that sensitivity to PARP inhibition also appears when other HR proteins besides BRCA1/2 are nonfunctional or lost [44,45].

This mechanistic interpretation of PARPi-associated synthetic lethality may, however, be a simplification of the underlying biological effect. It is now clear that PARP1 is involved in other DNA damage responses as well as SSBR, with reported functions in DNA replication and repair of stalled replication forks [46,47]. Moreover, certain PARP inhibitors may also have a direct cytotoxic effect by trapping PARP at DNA SSBs. These trapped PARP enzymes eventually induce replication fork stalling, which results in cell cycle arrest and apoptosis [48].

Lastly, of particular relevance to PC, PARP1 is involved in transcriptional regulation and has been implicated in AR

Please cite this article in press as: Mateo J, et al. DNA Repair in Prostate Cancer: Biology and Clinical Implications. Eur Urol (2016), http://dx.doi.org/10.1016/j.eururo.2016.08.037

signaling and ERG function [49,50]. This direct interaction between PARP1 and ERG, as well as an interaction between PI3K/PTEN pathway aberrations and HR DNA repair [51,52], also raised hopes for a wider target population. However, these mechanisms have not been confirmed in human clinical trials to date [53,54].

3.7. Clinical development of PARP inhibitors in PC

A first-in-man clinical trial of olaparib among a cohort of patients with advanced solid tumors enriched in gBRCA1/2 mutation carriers provided critical proof of concept and clinical data on the exquisite antitumor activity of this drug in BRCA-deficient tumors [55]. Since then, olaparib has been evaluated in several phase II/III studies, mainly in ovarian cancer as a single agent, until granted US Food and Drug Administration and European Medicines Agency approval in 2014 for advanced ovarian cancer associated with *BRCA1/2* mutations [56–59].

With regard to mCRPC patients, a few carriers of deleterious gBRCA1/2 mutations were enrolled in the initial trials of olaparib, and showed promising tumor responses. In a phase 2 basket trial including 298 g*BRCA1/2* mutation carriers with different tumor types, eight mCRPC patients were enrolled (1 *BRCA1* mutant carrier, 7 *BRCA2* cases) [60]. Half (4/8) of the mCRPC patients experienced a radiologic partial response; the median progression-free survival for all eight patients was 7.2 mo, with two patients responding for over 1 yr. Of note, 4/8 patients had prior treatment with platinum-based chemotherapy before receiving olaparib. In line with data suggesting some degree of secondary cross-resistance [61], only 1/4 patients who were exposed to platinum responded to olaparib, compared to 3/4 of those who were platinum-naïve.

Other PARP inhibitors are in clinical development; data for PC patients are primarily from g*BRCA1/2* mutation carriers with PC who participated in early clinical trials of these compounds. Preclinical studies of BMN673 (Biomarin/Medivation) demonstrated high potency in inhibiting PARP [62], and tumor responses were seen in *BRCA1/2* mutation carriers across tumor types in a phase 1 clinical trial [63]. Rucaparib (AG-014699/CO-338, Pfizer/Clovis Oncology) and veliparib (ABT-888, Abbott Laboratories) have mainly been developed so far in combination with chemotherapies or other targeted agents [64,65].

The antitumor activity of PARP inhibitors as single agents in patients besides g*BRCA1/2* mutation carriers has been investigated in two studies. During the first-in-man trial of niraparib (MK-4827, Merck/Tesaro), an expansion cohort for "sporadic" CRPC patients was pursued. Eighteen patients received niraparib at the recommended phase 2 dose (300 mg QD). One patient achieved a >50% decrease in PSA, remaining on treatment for 10 mo [54]. Three more patients had significant declines in circulating tumor cell (CTC) counts for >6 mo. The trial was unable to associate responses with either PTEN or ERG expression.

More recently, results from the first stage of a phase 2 investigator-initiated adaptive study of olaparib in mCRPC have been reported, raising interest in developing PARP inhibitors for this disease. The TOPARP study conducted in the UK included a first stage (TOPARP-A) aimed at testing the antitumor activity of olaparib in a "sporadic" mCRPC population (not known to be g*BRCA1/2* mutation carriers and not selected based on any prior knowledge of the genomic background) [66]. The primary endpoint of the study was the response rate, using a composite definition of response: radiologic response according to RECIST 1.1 and/or PSA declines >50% and/or conversion in CTC count from poor (>5 CTC/7.5 ml of blood) to positive prognostic profile (≤5 CTC/7.5 ml of blood), confirmed in at least two readings 4 wk apart. Progression-free and overall survival were explored as secondary endpoints. Response to olaparib was evaluated in 49/50 patients who received at least one dose of olaparib. These were all mCRPC patients progressing on docetaxel and, for all but one, on abiraterone and/or enzalutamide. Some 58% of patients also progressed on cabazitaxel before participating in the study. Of the 49 patients, 16 fulfilled at least one of the response criteria, including 11 cases with a PSA decline >50% and 6/32 with radiologic partial responses among the patients with measurable disease. The antitumor activity observed was strongly associated with the presence of mutations or homozygous deletions in DNA repair genes, evaluated by next-generation sequencing for metastatic biopsies collected at trial entry. Seven patients were found to have biallelic loss of *BRCA2*, either by germline or somatic mutations and deletions, with all seven responding to therapy. In five cases, mutations impacting *ATM* function were found; 4/5 responded to olaparib, including patients with germline and somatic mutations, and two patients with a single-allele mutation in the ATM kinase domain and no evidence of biallelic loss. Moreover, four cases with biallelic events in other genes involved in DNA damage response, including *PALB2*, *FANCA*, and *BRCA1*, showed benefit, primarily involving prolonged CTC conversions. Only two patients responding to olaparib did not have a clear DNA repair defect according to genomic analysis. Several long response durations were observed, including four patients benefiting for >1 yr. Patients with defects in DNA repair genes exhibited improved progression-free and overall survival from treatment initiation, although the preliminary survival data reported will need to be re-evaluated after longer follow-up.

The promising results in this first stage of the TOPARP study led to initiation of a second trial (TOPARP-B) with prospective selection of patients with aberrations in DNA repair genes; the objectives are to validate the antitumor activity seen in patients with the most common mutations (*BRCA2*, *ATM*) and to acquire critical data on sensitivity to olaparib for patients with mutations or deletions in less commonly affected genes.

The tolerability profile of PARP inhibitors is manageable, with anemia, thrombocytopenia, fatigue, and gastrointestinal toxicities (primarily nausea) the most frequent. In the TOPARP-A trial, anemia (20%) and fatigue (12%) were the most common grade ≥3 adverse events; gastrointestinal toxicities were less relevant than reported for ovarian cancer [67]. Hematologic toxicities and fatigue were also

Please cite this article in press as: Mateo J, et al. DNA Repair in Prostate Cancer: Biology and Clinical Implications. Eur Urol (2016), http://dx.doi.org/10.1016/j.eururo.2016.08.037

the dose-limiting events determining the recommended dose for other PARP inhibitors such as BMN673 and niraparib [54,63].

PARP inhibitors are also being evaluated in combination trials in mCRPC. An obvious strategy is to combine PARPi with DNA-damaging agents, mostly chemotherapy agents, to achieve a synergistic effect by blocking the response to chemotherapy-induced DNA damage. In a trial of veliparib and the alkylating agent temozolamide [65], 2/26 treated patients experienced PSA declines of >30%; the rate of grade 3–4 anemia and thrombocytopenia was 15% and 23% respectively. Overlapping hematologic toxicities also represent a major hurdle for combining platinum chemotherapies and PARPi.

An alternative approach would be to aim for a synthetic lethal interaction rather than a synergistic effect. Preclinical data demonstrating enhanced death of prostate tumor cells when combining HDAC and PARPi exemplify an opportunity for clinical development [68].

Lastly, trials combining PARPi with AR-targeting agents may be of interest on the basis of the crossregulation of both pathways and the central role of hormonal therapy in PC. Preliminary results from a randomized trial combining veliparib and abiraterone determined that 27% of patients had aberrations in DNA repair genes; this subgroup experienced high response rates to the combination and, remarkably, to abiraterone alone [53]. Data from a randomized trial combining abiraterone and olaparib are also expected. However, interpretation of putative predictive biomarkers of response in combination trials may be challenging.

3.8. *DNA damaging agents: should they be reconsidered for PC?*

Platinum salts are part of standard management for other tumor types, but their use in PC has been limited since phase 3 trials of the orally available platinum derivative satraplatin failed to meet the primary endpoint of overall survival (OS) improvement [69]. However, some antitumor activity has been described for carboplatin, cisplatin, and satraplatin in mCRPC. This, together with the possibility now of identifying DNA repair–defective tumors and data on DNA repair mutations and response to platinum from ovarian cancer studies, has raised interest in re-evaluating the role of platinum agents in this disease.

Recently, Kumar et al reported longer benefit from carboplatin for cases with HR defects in a retrospective series of patients ($p = 0.002$ for duration of treatment, $n = 21$). Small case series have reported tumor responses to carboplatin in mCRPC patients with biallelic *BRCA2* loss [70]. Nonetheless, the mechanisms involved in sensitivity to platinum and PARPi may be similar but not identical, and further investigation of cross-sensitivity and cross-resistance between agents is now needed following data from ovarian cancer studies. For example, the predominance of NER in repairing platinum-generated adducts warrants specific clinical trials [71,72].

A few clinical trials have explored combinations of carboplatin and taxanes for PC. One of the most relevant was a phase 2 study of carboplatin and docetaxel, followed by cisplatin and etoposide on progression. The study recruited 120 patients with mCRPC with prespecified clinicopathologic characteristics suggestive of more aggressive, arguably less AR-dependent disease [73]. With median OS of 16 mo, the radiological response rate was ~30% for both first- and second-line combinations. The tolerability was relatively acceptable, with only three cases of febrile neutropenia.

Use of the topoisomerase inhibitor mitoxantrone in PC has declined as several other therapies became available over the last decade. However, the main mechanism in the cytotoxicity of mitoxantrone is disruption of DNA synthesis and repair, so re-evaluation of its activity in molecularly defined populations may be of interest.

4. Conclusions

The identification of a subgroup of PCs with lethal disease with genomic deleterious aberrations of DNA repair genes supports further evaluation of this biomarker-driven treatment stratification of advanced PC in registration studies. If the efficacy of this strategy is, it might also be possible to apply it to earlier disease stages, including high-risk locally advanced disease.

Further studies are now needed to clinically qualify multiplex predictive biomarkers of DNA repair–defective PCs, particularly for the less common genomic aberrations that cause this phenotype. On the basis of recent studies indicating that these aberrations are common in the germline DNA of patients with metastatic PC, somatic and germline DNA testing for patients with advanced PC should be considered in view not only of the therapeutic consequences for the patient but also the possibility of pursuing targeted screening in this population. A major limitation at present for adoption of this strategy is the implementation and standardization of genomic testing in the community setting, but the decreasing costs of next-generation sequencing and lessons learned from stratified therapies in other diseases will help us to pursue more precise care for PC patients.

Author contributions: Johann S. de Bono had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Mateo, Boysen, de Bono.
Acquisition of data: Mateo, Boysen, de Bono.
Analysis and interpretation of data: All authors.
Drafting of the manuscript: Mateo, Boysen.
Critical revision of the manuscript for important intellectual content: All authors.
Statistical analysis: None.
Obtaining funding: None.
Administrative, technical, or material support: None.
Supervision: Barbieri, Bryant, Castro, Nelson, Olmos, Pritchard, Rubin, de Bono.
Other: None.

Financial disclosures: Johann S. de Bono certifies that all conflicts of interest, including specific financial interests and relationships and

Please cite this article in press as: Mateo J, et al. DNA Repair in Prostate Cancer: Biology and Clinical Implications. Eur Urol (2016), http://dx.doi.org/10.1016/j.eururo.2016.08.037

affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

Funding/Support and role of the sponsor: None.

Acknowledgments: J. Mateo and J.S. de Bono acknowledge funding from an Experimental Cancer Medical Centre grant, Cancer Research UK, Prostate Cancer Foundation, Prostate Cancer UK, a Biomedical Research Centre grant to the ICR/Royal Marsden, and a Medical Research Council - Prostate Cancer UK - Movember Fellowship to J. Mateo. G. Boysen was supported by a Marie Sklodowska-Curie International Individual Postdoctoral Fellowship (CDELP) and Prostate Cancer UK (PG13-036). C.E. Barbieri is a Damon Runyon Clinical Investigator supported (in part) by the Damon Runyon Cancer Research Foundation, and supported by the US National Cancer Institute (K08CA187417-02), the Prostate Cancer Foundation, and the Urology Care Foundation (Rising Star in Urology Research Award). H. Bryant is supported by Yorkshire Cancer Research (SS012PHD). C.C. Pritchard is supported by a CDMRP award (PC131820). The Prostate Cancer Unit at CNIO is supported by CRIS Fundación contra el cancer. E. Castro is the recipient of a Juan de la Cierva Grant from Ministerio de Economía y Competitividad (Spain). D. Olmos is also supported by Fundación Científica de la Asociación Española Contra el Cáncer. The Prostate Cancer Foundation provided support to the following authors through Young Investigator Awards: J. Mateo, C.E. Barbieri, D. Olmos, and C.C. Pritchard.

References

[1] Lorente D, Mateo J, Perez-Lopez R, de Bono JS, Attard G. Sequencing of agents in castration-resistant prostate cancer. Lancet Oncol 2015;16:e279–92.
[2] Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001;344:783–92.
[3] Flaherty KT, Robert C, Hersey P, et al. Improved survival with MEK inhibition in BRAF-mutated melanoma. N Engl J Med 2012;367:107–14.
[4] Rubin MA, Girelli G, Demichelis F. Genomic correlates to the newly proposed grading prognostic groups for prostate cancer. Eur Urol 2015;69:2–5.
[5] Baca SC, Prandi D, Lawrence MS, et al. Punctuated evolution of prostate cancer genomes. Cell 2013;153:666–77.
[6] Barbieri CE, Baca SC, Lawrence MS, et al. Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. Nat Genet 2012;44:685–9.
[7] Berger MF, Lawrence MS, Demichelis F, et al. The genomic complexity of primary human prostate cancer. Nature 2011;470:214–20.
[8] Abeshouse A, Ahn J, Akbani R, et al. The molecular taxonomy of primary prostate cancer. Cell 2015;163:1011–25.
[9] Meeks HD, Song H, Michailidou K, et al. BRCA2 polymorphic stop codon K3326X and the risk of breast, prostate, and ovarian cancers. J Natl Cancer Inst 2016;108, djv315.
[10] Beltran H, Yelensky R, Frampton GM, et al. Targeted next-generation sequencing of advanced prostate cancer identifies potential therapeutic targets and disease heterogeneity. Eur Urol 2013;63:920–6.
[11] Grasso CS, Wu Y-M, Robinson DR, et al. The mutational landscape of lethal castration-resistant prostate cancer. Nature 2012;487:239–43.
[12] Robinson D, Van Allen EM, Wu Y-M, et al. Integrative clinical genomics of advanced prostate cancer. Cell 2015;161:1215–28.
[13] Kumar A, Coleman I, Morrissey C, et al. Substantial interindividual and limited intraindividual genomic diversity among tumors from men with metastatic prostate cancer. Nat Med 2016;22:369–78.
[14] Beltran H, Prandi D, Mosquera JM, et al. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. Nat Med 2016;22:298–305.
[15] Gundem G, Van Loo P, Kremeyer B, et al. The evolutionary history of lethal metastatic prostate cancer. Nature 2015;520:353–7.
[16] Wood RD, Wood RD, Mitchell M, Sgouros J, Lindahl T. Human DNA repair genes. Science 2011;1284:241–83.
[17] Saleh-Gohari N, Bryant HE, Schultz N, Parker KM, Cassel TN, Helleday T. Spontaneous homologous recombination is induced by collapsed replication forks that are caused by endogenous DNA single-strand breaks. Mol Cell Biol 2005;25:7158–69.
[18] Goodwin JF, Schiewer MJ, Dean JL, et al. A hormone-DNA repair circuit governs the response to genotoxic insult. Cancer Discov 2013;3:1254–71.
[19] Polkinghorn WR, Parker JS, Lee MX, et al. Androgen receptor signaling regulates DNA repair in prostate cancers. Cancer Discov 2013;3:1245–53.
[20] Lin C, Yang L, Tanasa B, et al. Nuclear receptor-induced chromosomal proximity and DNA breaks underlie specific translocations in cancer. Cell 2009;139:1069–83.
[21] Ju B-G, Lunyak VV, Perissi V, et al. A topoisomerase IIβ-mediated dsDNA break required for regulated transcription. Science 2006;312:1798–802.
[22] Wu C, Wyatt AW, McPherson A, et al. Poly-gene fusion transcripts and chromothripsis in prostate cancer. Genes Chromosomes Cancer 2012;51:1144–53.
[23] Tomlins SA, Rhodes DR, Perner S, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 2005;310:644–8.
[24] Mani R, Tomlins SA, Callahan K, et al. Induced chromosomal proximity and the genesis of gene fusions in prostate cancer. Science 2010;326:2009–11.
[25] Boysen G, Barbieri CE, Prandi D, et al. SPOP mutation leads to genomic instability in prostate cancer. Elife 2015;4:e09207.
[26] Pritchard CC, Morrissey C, Kumar A, et al. Complex MSH2 and MSH6 mutations in hypermutated microsatellite unstable advanced prostate cancer. Nat Commun 2014;5:4988.
[27] Chen Y, Wang J, Fraig MM, et al. Defects of DNA mismatch repair in human prostate cancer. Cancer Res 2001;61:4112–21.
[28] Decker B, Karyadi DM, Davis BW, et al. Biallelic BRCA2 mutations shape the somatic mutational landscape of aggressive prostate tumors. Am J Hum Genet 2016;98:1–12.
[29] Castro E, Jugurnauth-Little S, Karlsson Q, et al. High burden of copy number alterations and c-MYC amplification in prostate cancer from BRCA2 germline mutation carriers. Ann Oncol 2015;26:2293–300.
[30] Carter BS, Beaty TH, Steinberg GD, Childs B, Walsh PC. Mendelian inheritance of familial prostate cancer. Proc Natl Acad Sci U S A 1992;89:3367–71.
[31] Kote-Jarai Z, Leongamornlert D, Saunders E, et al. BRCA2 is a moderate penetrance gene contributing to young-onset prostate cancer: implications for genetic testing in prostate cancer patients. Br J Cancer 2011;105:1230–4.
[32] Leongamornlert D, Mahmud N, Tymrakiewicz M, et al. Germline BRCA1 mutations increase prostate cancer risk. Br J Cancer 2012;106:1697–701.
[33] Eeles R, Goh C, Castro E, et al. The genetic epidemiology of prostate cancer and its clinical implications. Nat Rev Urol 2014;11:18–31.
[34] Pritchard CC, Mateo J, Walsh MF, et al. Inherited DNA repair gene mutations in men with metastatic prostate cancer. N Engl J Med 2016;375:443–5. http://dx.doi.org/10.1056/NEJMoa1603144.
[35] Bancroft EK, Page EC, Castro E, et al. Targeted prostate cancer screening in BRCA1 and BRCA2 mutation carriers: results from the initial screening round of the IMPACT study. Eur Urol 2014;66:489–99.

[36] Haraldsdottir S, Hampel H, Wei L, et al. Prostate cancer incidence in males with Lynch syndrome. Genet Med 2014;16:553–7.
[37] Castro E, Goh C, Olmos D, et al. Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer. J Clin Oncol 2013;31:1748–57.
[38] Castro E, Goh C, Leongamornlert D, et al. Effect of BRCA mutations on metastatic relapse and cause-specific survival after radical treatment for localised prostate cancer. Eur Urol 2015;68:186–93.
[39] Gallagher DJ, Cronin AM, Milowsky MI, et al. Germline BRCA mutation does not prevent response to taxane-based therapy for the treatment of castration-resistant prostate cancer. BJU Int 2012;109:713–9.
[40] Gallagher DJ, Gaudet MM, Pal P, et al. Germline BRCA mutations denote a clinicopathologic subset of prostate cancer. Clin Cancer Res 2010;16:2115–21.
[41] Dobzhansky T. Genetics of natural populations. Xiii. Recombination and variability in populations of Drosophila pseudoobscura. Genetics 1946;31:269–90.
[42] Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 2005;434:917–21.
[43] Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 2005;434:913–7.
[44] McCabe N, Turner NC, Lord CJ, et al. Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(-ADP-ribose) polymerase inhibition. Cancer Res 2006;66:8109–15.
[45] Murai J, Huang S-YN, Renaud A, et al. Stereospecific PARP trapping by BMN 673 and comparison with olaparib and rucaparib. Mol Cancer Ther 2014;13:433–43.
[46] Patel AG, Sarkaria JN, Kaufmann SH. Nonhomologous end joining drives poly(ADP-ribose) polymerase (PARP) inhibitor lethality in homologous recombination-deficient cells. Proc Natl Acad Sci U S A 2011;108:3406–11.
[47] Bryant HE, Petermann E, Schultz N, et al. PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. EMBO J 2009;28:2601–15.
[48] Murai J, Huang SN, Das BB. Trapping of PARP1 and PARP2 by clinical PARP inhibitors. Cancer Res 2012;72:5588–99.
[49] Schiewer MJ, Goodwin JF, Han S, et al. Dual roles of PARP-1 promote cancer growth and progression. Cancer Discov 2012;2:1134–49.
[50] Brenner JC, Ateeq B, Li Y, et al. Mechanistic rationale for inhibition of poly(ADP-ribose) polymerase in ETS gene fusion-positive prostate cancer. Cancer Cell 2011;19:664–78.
[51] Kumar A, Fernandez-Capetillo O, Carrera AC. Nuclear phosphoinositide 3-kinase controls double-strand break DNA repair. Proc Natl Acad Sci U S A 2010;107:7491–6.
[52] McCabe N, Hanna C, Walker SM, et al. Mechanistic rationale to target PTEN-deficient tumor cells with inhibitors of the DNA damage response kinase ATM. Cancer Res 2015;75:2159–65.
[53] Hussain M, Daignault S, Twardowski P, et al. Co-targeting androgen receptor (AR) and DNA repair: a randomized ETS gene fusion-stratified trial of abiraterone + prednisone (Abi) +/− the PARP1 inhibitor veliparib for metastatic castration-resistant prostate cancer (mCRPC) patients (pts) (NCI9012). J Clin Oncol 2016;34(Suppl):5010.
[54] Sandhu SK, Schelman WR, Wilding G, et al. The poly(ADP-ribose) polymerase inhibitor niraparib (MK4827) in BRCA mutation carriers and patients with sporadic cancer: a phase 1 dose-escalation trial. Lancet Oncol 2013;2045:1–11.
[55] Fong PCP, Boss DDS, Yap T, et al. Inhibition of poly (ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med 2009;361:123–34.
[56] Ledermann J, Harter P, Gourley C, et al. Olaparib maintenance therapy in platinum-sensitive relapsed ovarian cancer. N Engl J Med 2012;366:1382–92.
[57] Tutt A, Robson M, Garber JE, et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. Lancet 2010;376:235–44.
[58] Ledermann J, Harter P, Gourley C, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. Lancet Oncol 2014;15:852–61.
[59] Audeh MW, Carmichael J, Penson RT, et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: a proof-of-concept trial. Lancet 2010;376:245–51.
[60] Kaufman B, Shapira-Frommer R, Schmutzler RK, et al. Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. J Clin Oncol 2015;33:244–50.
[61] Fong PC, Yap T, Boss DS, et al. Poly(ADP)-ribose polymerase inhibition: frequent durable responses in BRCA carrier ovarian cancer correlating with platinum-free interval. J Clin Oncol 2010;28:2512–9.
[62] Shen Y, Rehman FL, Feng Y, et al. BMN 673, a novel and highly potent PARP1/2 inhibitor for the treatment of human cancers with DNA repair deficiency. Clin Cancer Res 2013;19:5003–15.
[63] de Bono JS, Mina L, Gonzalez M, et al. First-in-human trial of novel oral PARP inhibitor BMN 673 in patients with solid tumors. J Clin Oncol 2013;31(Suppl):2580.
[64] Ihnen M, zu Eulenburg C, Kolarova T, et al. Therapeutic potential of the poly(ADP-ribose) polymerase inhibitor rucaparib for the treatment of sporadic human ovarian cancer. Mol Cancer Ther 2013;12:1002–15.
[65] Hussain M, Carducci MA, Slovin S, et al. Targeting DNA repair with combination veliparib (ABT-888) and temozolomide in patients with metastatic castration-resistant prostate cancer. Invest New Drugs 2014;32:904–12.
[66] Mateo J, Carreira S, Sandhu S, et al. DNA-repair defects and olaparib in metastatic prostate cancer. N Engl J Med 2015;373:1697–708.
[67] Mateo J, Moreno V, Gupta A, et al. An adaptive study to determine the optimal dose of the tablet formulation of the PARP inhibitor olaparib. Target Oncol 2016;11:401–15.
[68] Chao OS, Goodman OB. Synergistic loss of prostate cancer cell viability by coinhibition of HDAC and PARP. Mol Cancer Res 2014;12:1755–66.
[69] Sternberg CN, Petrylak DP, Sartor O, et al. Multinational, double-blind, phase III study of prednisone and either satraplatin or placebo in patients with castrate-refractory prostate cancer progressing after prior chemotherapy: the SPARC trial. J Clin Oncol 2009;27:5431–8.
[70] Cheng HH, Pritchard CC, Boyd T, Nelson PS, Montgomery B. Biallelic inactivation of BRCA2 in platinum-sensitive metastatic castration-resistant prostate cancer. Eur Urol 2016;69:992–5.
[71] Ang JE, Gourley C, Powell B. Efficacy of chemotherapy in BRCA1/2 mutation carrier ovarian cancer in the setting of poly(ADP-ribose) polymerase inhibitor resistance: a multi-institutional study. Clin Cancer Res 2013;44:1–31.
[72] Ceccaldi R, O'Connor KW, Mouw KW, et al. A unique subset of epithelial ovarian cancers with platinum sensitivity and PARP inhibitor resistance. Cancer Res 2015;75:1–8.
[73] Aparicio AM, Harzstark AL, Corn PG, et al. Platinum-based chemotherapy for variant castrate-resistant prostate cancer. Clin Cancer Res 2013;19:3621–30.

Please cite this article in press as: Mateo J, et al. DNA Repair in Prostate Cancer: Biology and Clinical Implications. Eur Urol (2016), http://dx.doi.org/10.1016/j.eururo.2016.08.037

ORIGINAL ARTICLE

Inherited DNA-Repair Gene Mutations in Men with Metastatic Prostate Cancer

C.C. Pritchard, J. Mateo, M.F. Walsh, N. De Sarkar, W. Abida, H. Beltran, A. Garofalo, R. Gulati, S. Carreira, R. Eeles, O. Elemento, M.A. Rubin, D. Robinson, R. Lonigro, M. Hussain, A. Chinnaiyan, J. Vinson, J. Filipenko, L. Garraway, M.-E. Taplin, S. AlDubayan, G.C. Han, M. Beightol, C. Morrissey, B. Nghiem, H.H. Cheng, B. Montgomery, T. Walsh, S. Casadei, M. Berger, L. Zhang, A. Zehir, J. Vijai, H.I. Scher, C. Sawyers, N. Schultz, P.W. Kantoff, D. Solit, M. Robson, E.M. Van Allen, K. Offit, J. de Bono, and P.S. Nelson

ABSTRACT

BACKGROUND

Inherited mutations in DNA-repair genes such as *BRCA2* are associated with increased risks of lethal prostate cancer. Although the prevalence of germline mutations in DNA-repair genes among men with localized prostate cancer who are unselected for family predisposition is insufficient to warrant routine testing, the frequency of such mutations in patients with metastatic prostate cancer has not been established.

METHODS

We recruited 692 men with documented metastatic prostate cancer who were unselected for family history of cancer or age at diagnosis. We isolated germline DNA and used multiplex sequencing assays to assess mutations in 20 DNA-repair genes associated with autosomal dominant cancer-predisposition syndromes.

RESULTS

A total of 84 germline DNA-repair gene mutations that were presumed to be deleterious were identified in 82 men (11.8%); mutations were found in 16 genes, including *BRCA2* (37 men [5.3%]), *ATM* (11 [1.6%]), *CHEK2* (10 [1.9% of 534 men with data]), *BRCA1* (6 [0.9%]), *RAD51D* (3 [0.4%]), and *PALB2* (3 [0.4%]). Mutation frequencies did not differ according to whether a family history of prostate cancer was present or according to age at diagnosis. Overall, the frequency of germline mutations in DNA-repair genes among men with metastatic prostate cancer significantly exceeded the prevalence of 4.6% among 499 men with localized prostate cancer ($P<0.001$), including men with high-risk disease, and the prevalence of 2.7% in the Exome Aggregation Consortium, which includes 53,105 persons without a known cancer diagnosis ($P<0.001$).

CONCLUSIONS

In our multicenter study, the incidence of germline mutations in genes mediating DNA-repair processes among men with metastatic prostate cancer was 11.8%, which was significantly higher than the incidence among men with localized prostate cancer. The frequencies of germline mutations in DNA-repair genes among men with metastatic disease did not differ significantly according to age at diagnosis or family history of prostate cancer. (Funded by Stand Up To Cancer and others.)

The authors' full names, academic degrees, and affiliations are listed in the Appendix. Address reprint requests to Dr. Nelson at the Division of Human Biology, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave., Mailstop D4-100, Seattle, WA 98109, or at pnelson@fhcrc.org.

Drs. Pritchard, Mateo, and Walsh and Drs. Offit, de Bono, and Nelson contributed equally to this article.

This article was published on July 6, 2016, at NEJM.org.

DOI: 10.1056/NEJMoa1603144

Copyright © 2016 Massachusetts Medical Society.

The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

Carcinoma of the prostate is a common cancer with a wide spectrum of clinical behavior that ranges from decades of indolence to rapid metastatic progression and lethality.[1,2] Prostate cancer is also among the most heritable of human cancers, with 57% (95% confidence interval [CI], 51 to 63) of the interindividual variation in risk attributed to genetic factors.[3] Thus far, genomewide association studies have identified more than 100 common variants that account for approximately 33% of the excess familial prostate cancer risk.[4-7] Mutations in other genes, including *BRCA1*, *BRCA2*, *MSH2*,[8-10] and *HOXB13*,[11] account for a small proportion of familial cases, with *BRCA2* mutations associated with 1.2 to 1.8% of prostate cancer overall.[9,12]

Thus far, only mutations that disrupt the function of genes involved in repairing DNA damage through homologous recombination have been shown to be associated with the aggressive clinical behavior of localized prostate cancer and with cancer-specific mortality.[9,12-14] The need for genetic prognostic markers is critical, because the clinicopathological diversity of prostate cancer has confounded efforts to develop effective screening strategies that avoid overdetection and overtreatment yet capture cancers that are destined to affect survival.[15] Persons who are shown to have cancer-predisposition mutations in the germline may serve as sentinels for the identification of families at high risk. It should be noted that men with metastatic prostate cancer and DNA-repair gene mutations have been reported to have sustained responses to poly-ADP ribose polymerase (PARP) inhibitors and platinum-based chemotherapy.[16,17]

Although the prevalence of germline DNA-repair gene mutations is low among men with localized prostate cancer who are unselected for family predisposition, the frequency of such mutations among men with metastatic prostate cancer has not been established. We recently reported an analysis of the spectrum of somatic aberrations that occur in metastatic prostate cancer, using whole-exome sequencing of metastatic tumors.[18] For comparison purposes, we also sequenced germline DNA exomes from these men and unexpectedly found that 8% carried pathogenic germline mutations in DNA-repair genes. This finding suggested that men with metastatic prostate cancer represent a population that is enriched for heritable defects in DNA repair. To confirm this finding and to further ascertain the spectrum and prevalence of germline DNA-repair gene mutations in metastatic prostate cancer, we recruited 542 additional men with a confirmed prostate cancer metastasis and used next-generation sequencing to analyze DNA-repair genes associated with autosomal dominant cancer-predisposition syndromes.

METHODS

STUDY POPULATIONS

Seven case series of men with metastatic prostate cancer across multiple institutions in the United States and United Kingdom, including a total of 692 patients, were analyzed. All the patients had a diagnosis of metastatic prostate cancer and were not selected on the basis of family history, age, or any knowledge of genetic background. The demographic characteristics of the men in each series are summarized in Table 1. Detailed information on the specific germline mutations and on clinical features of mutation carriers in each series is provided in Tables S1, S2, and S3 in the Supplementary Appendix, available with the full text of this article at NEJM.org.

Case Series 1, the Stand Up to Cancer–Prostate Cancer Foundation (SU2C-PCF) International Prostate Cancer Dream Team discovery series, was made up of 150 patients for whom data were previously reported in the SU2C-PCF study of molecular stratification of metastatic prostate cancer.[18] Case Series 2, the SU2C-PCF validation series, was made up of 84 patients who were newly enrolled in the SU2C-PCF study and for whom data had not been reported previously. Case Series 3, Royal Marsden Prostate Cancer Genomics series, included 131 patients who were considered for enrollment in clinical trials at the Royal Marsden Hospital from January 2013 through July 2015. Case Series 4 consisted of 91 consecutive patients included in the University of Washington rapid autopsy program from 1997 through 2013. Case Series 5 included 69 consecutive patients who were enrolled in the Weill Cornell Medical College precision medicine program. Case Series 6 was made up of 43 consecutive patients from the University of Michigan rapid autopsy program. Case Series 7, from the Memorial Sloan Kettering Cancer Center,

The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

included 124 consecutive patients who were enrolled through the Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) study.

The protocols for these case series were approved by the local institutional review boards, and written informed consent was obtained from all patients at the local sites before enrollment. Correlative clinical data were collected at each site with the use of electronic patient records and were entered into deidentified databases. The study was designed by the Stand Up To Cancer–Prostate Cancer Foundation International Prostate Cancer Dream Team investigators. The study sponsors had no role in the design of the study, the collection or analysis of the data, or the preparation of the manuscript. The manuscript was written by four of the authors. All authors reviewed the manuscript, agreed to submit the manuscript for publication, and vouch for the accuracy and completeness of the data and for the fidelity of the study to the protocol.

SEQUENCING AND BIOINFORMATICS ANALYSIS

For the analysis involving Case Series 1, 2, and 6, whole-exome sequencing of germline and tumor DNA was performed as described previously.[18] Germline DNA from buccal swabs, buffy coats, or whole blood was isolated with the use of the QIAGEN DNeasy Blood and Tissue Kit. Whole-exome sequencing was performed on the Illumina HiSeq 2500 in paired-end mode.

For the analysis of Case Series 3, germline DNA was extracted from saliva or buccal swab samples with the use of the Oragene kit (DNA Genotek). Libraries for targeted sequencing were constructed with a customized GeneRead DNaseq Panel (Qiagen) covering 53 genes and run on the Illumina MiSeq sequencer, as described previously.[16]

For the analyses of Case Series 4 and 5, germline DNA was extracted from peripheral blood or nontumor tissue and from matched tumor DNA, as described previously.[19] Targeted deep sequencing was performed with the BROCA panel of 53 DNA-repair pathway genes. The bioinformatics pipeline has been described previously.[20,21] For tumors from Case Series 5, analyses were performed by means of exome sequencing, as described previously.[22] For Case Series 7, tumor and germline genomic sequencing was performed as described previously, with the use of the MSK-IMPACT hybrid capture-based next-generation sequencing assay.[23,24]

The mean sequencing depth of coverage was more than 100× for all case series, with the exception of sequencing of *BAP1*, *BARD1*, *BRIP1*, and *FAM175A*, which were not included on the Royal Marsden Hospital panel, and *GEN1*, which was not included on the Royal Marsden Hospital or Memorial Sloan Kettering panel. Data from the Royal Marsden Hospital and Memorial Sloan Kettering cases were censored for analyses of these genes. In addition, data were censored for *CHEK2* in 158 cases for which exon sequencing coverage was incomplete. The depth of coverage for each gene according to site is provided in Table S4 in the Supplementary Appendix.

To compare our results with data from a large series of patients with localized prostate cancer, we analyzed public data from the Cancer Genome Atlas prostate cancer study.[25] Paired-end reads (100 bp) were aligned to the hg19 reference human genome with the use of the Burrows–Wheeler Aligner. Annotations were defined with ANNOVAR (http://annovar.openbioinformatics.org/en/latest). Population allele frequencies were extracted from the Exome Aggregation Consortium ExAC Browser (http://exac.broadinstitute.org/), 1000 Genomes (www.1000genomes.org), and the single-nucleotide polymorphism database of the National Center for Biotechnology Information (dbSNP), version 138 (www.ncbi.nlm.nih.gov/projects/SNP).

INTERPRETATION OF VARIANTS

Our analysis focused on variants identified among 20 genes associated with autosomal dominant cancer-predisposition syndromes that involve maintenance of DNA integrity (Table 2). The pathogenicity of germline variants was determined according to established American College of Medical Genetics and Genomics and Association for Molecular Pathology consensus criteria and International Agency for Research on Cancer guidelines.[24,26] At least two independent expert reviewers evaluated all variants against published literature and public databases, including ClinVar and variant-specific databases, in addition to population frequency databases, including 1000 Genomes and the Exome Aggregation Consortium. Expected high-penetrance or moderate-penetrance variants classified as mutations that are pathogenic or likely

The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

Table 1. Demographic Characteristics of the Patients with Prostate Cancer.*

Characteristic	Case Series 1 (N=150)		Case Series 2 (N=84)		Case Series 3 (N=131)		Case Series 4 (N=91)		Case Series 5 (N=69)		Case Series 6 (N=43)		Case Series 7 (N=124)		All Case Series (N=692)		TCGA Cohort with Primary Prostate Cancer (N=499)†			
																	L-I Risk		High Risk	
	M	NM	M	NM	M	NM	M	NM	M	NM	M	NM	M	NM	M	NM	M	NM	M	NM
	number of patients																			
Total	15	135	9	75	16	115	8	83	7	62	4	39	23	101	82	610	4	158	19	318
Age																				
<50 yr	2	16	0	2	1	6	1	5	1	2	0	3	2	9	7	43	0	15	1	11
50–59 yr	6	40	4	30	3	37	2	18	2	15	0	6	9	47	26	193	3	63	8	103
60–69 yr	6	63	3	24	11	56	3	38	3	20	2	25	11	36	39	262	1	67	8	167
70–79 yr	1	15	1	11	1	15	2	19	1	11	2	5	1	9	9	85	0	13	2	37
≥80 yr	0	1	0	2	0	1	0	1	0	8	0	0	0	0	0	13	0	0	0	0
Unknown	0	0	1	6	0	0	0	2	0	6	0	0	0	0	1	14	0	0	0	0
Race or ethnic background‡																				
Non-Hispanic white	12	116	8	60	16	103	5	70	3	37	4	30	22	90	70	506	2	118	16	270
Hispanic	0	1	1	0	0	0	0	0	2	7	0	0	0	0	3	8	0	1	1	5
Non-Hispanic black	1	8	0	4	0	12	2	0	1	5	0	2	0	5	4	36	2	27	2	27
Asian or Pacific Islander	1	2	0	2	0	0	0	0	0	5	0	1	0	1	1	11	0	3	0	9
Other or unknown	1	8	0	9	0	0	1	13	1	8	0	6	1	5	4	49	0	9	0	7
History of cancer in a first-degree relative																				
Prostate cancer	4	31	3	11	2	16	0	14	2	12	0	4	5	29	16	117	NA	NA	NA	NA
Other cancer	10	55	4	35	8	53	6	30	3	14	1	19	19	64	51	270	NA	NA	NA	NA
No cancer	2	46	2	27	5	35	1	25	3	29	1	12	4	25	18	199	NA	NA	NA	NA
Unknown	2	15	1	5	2	17	1	21	1	9	2	6	0	0	9	73	NA	NA	NA	NA
Gleason score§																				

The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

≤6	0	8	0	3	0	11	1	9	1	3	0	2	0	4	2	40	1	32	1	11
3+4	0	19	1	9	0	10	1	6	1	3	0	2	2	11	5	60	3	94	1	50
4+3	1	18	2	7	1	11	0	15	2	7	0	4	4	20	10	82	0	32	5	64
8–10	11	70	3	44	12	65	6	39	3	28	4	16	17	64	56	326	0	0	12	193
Unknown	3	20	3	12	3	18	0	14	0	21	0	15	0	2	9	102	0	0	0	0

* The studies included in each case series were as follows: 1, Stand Up To Cancer–Prostate Cancer Foundation discovery series; 2, Stand Up To Cancer–Prostate Cancer Foundation validation series; 3, Royal Marsden Hospital; 4, University of Washington; 5, Weill Cornell Medical College; 6, University of Michigan; and 7, Memorial Sloan Kettering Cancer Center. L-I denotes low to intermediate, M mutation present, NA not applicable, and NM no mutation present.
† The Cancer Genome Atlas (TCGA) cohort includes a series of patients with localized prostate cancer.
‡ Race and ethnic background were self-reported.
§ The Gleason score is a measure of the differentiation state of prostate cancer; scores range from 2 to 10, with higher scores associated with worse clinical outcomes. When two grades are given (e.g., 3+4), the first indicates the more common grade found in the tumor.

to be pathogenic are reported here. Low-penetrance variants, such as *CHEK2* p.I157T, were excluded.

STATISTICAL ANALYSIS

Associations between DNA-repair gene mutation status and age, race, or Gleason score strata were evaluated with the use of two-sided Fisher's exact tests. The frequencies of DNA-repair gene mutations among the 692 patients with metastatic prostate cancer were evaluated relative to the expected frequencies from the Exome Aggregation Consortium (53,105 persons) or the Cancer Genome Atlas cohort (499 persons) with the use of two-sided exact binomial tests. We also performed analyses in which the 150 men from the previously reported Case Series 1 were excluded[18] (Table S5 in the Supplementary Appendix). No adjustments were made for multiple comparisons; P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

PATIENT CHARACTERISTICS

All 692 men in our analysis had documented metastatic prostate cancer, as determined by histologic evaluation of a tumor-biopsy specimen or surgical-resection specimen. The demographic characteristics of the men from each case series are shown in Table 1.

GERMLINE DNA-REPAIR GENE MUTATIONS

We assessed 20 genes that maintain DNA integrity and have been associated with autosomal dominant cancer-predisposition syndromes (Table 2), using whole-exome sequencing or targeted next-generation sequencing assays designed to interrogate the status of DNA-repair genes.[27] Of the 692 men evaluated, 82 (11.8%) had at least one presumed pathogenic germline mutation in a gene involved in DNA-repair processes (Table 2). Mutation frequencies were similar across independent case series (Table 3). The 84 germline mutations that were presumed to be pathogenic (2 men had mutations in 2 genes) included 79 truncating mutations and 5 known deleterious missense mutations (Fig. 1, and Table S1 in the Supplementary Appendix). Mutations were identified in 16 different genes, including *BRCA2* (37 mutations [44% of total mutations]), *ATM* (11 [13%]), *CHEK2* (10 [12%]), *BRCA1* (6 [7%]), *RAD51D*

The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

Table 2. Germline Mutations in Metastatic Cases as Compared with the General Population and Primary Cases.

Gene	Metastatic Prostate Cancer (N=692)*	Exome Aggregation Consortium (N=53,105)†	TCGA Cohort with Primary Prostate Cancer (N=499)	Metastatic Prostate Cancer vs. Exome Aggregation Consortium		Metastatic Prostate Cancer vs. TCGA Cohort	
	No. of Mutations (% of Men)			Relative Risk (95% CI)	P Value	Relative Risk (95% CI)	P Value
ATM	11 (1.59)	133 (0.25)	5 (1.00)	6.3 (3.2–11.3)	<0.001	1.6 (0.8–2.8)	0.12
ATR	2 (0.29)	43 (0.08)	0	3.6 (0.4–12.8)	0.11	—	—
BAP1‡	0	1	0	—	—	—	—
BARD1‡	0	38 (0.07)	1 (0.20)	—	—	—	—
BRCA1	6 (0.87)	104 (0.22)	3 (0.60)	3.9 (1.4–8.5)	0.005	1.4 (0.5–3.1)	0.32
BRCA2	37 (5.35)	153 (0.29)	1 (0.20)	18.6 (13.2–25.3)	<0.001	26.7 (18.9–36.4)	<0.001
BRIP1‡	1 (0.18)	100 (0.19)	1 (0.20)	0.9 (0.02–5.3)	1.0	0.9 (0.0–4.9)	1.0
CHEK2‡	10 (1.87)	314 (0.61)	2 (0.40)	3.1 (1.5–5.6)	0.002	4.7 (2.2–8.5)	<0.001
FAM175A‡	1 (0.18)	52 (0.10)	0	1.8 (0.05–10.1)	0.42	—	—
GEN1‡	2 (0.46)	42 (0.08)	0	5.8 (0.7–20.8)	0.048	—	—
MLH1	0	11 (0.02)	0	—	—	—	—
MRE11A	1 (0.14)	36 (0.07)	1 (0.20)	2.1 (0.1–11.8)	0.38	0.7 (0.0–4.0)	1.0
MSH2	1 (0.14)	23 (0.04)	1 (0.20)	3.3 (0.1–18.5)	0.26	0.7 (0.0–4.0)	1.0
MSH6	1 (0.14)	41 (0.08)	1 (0.20)	1.9 (0.05–10.4)	0.41	0.7 (0.0–4.0)	1.0
NBN	2 (0.29)	61 (0.11)	1 (0.20)	2.5 (0.3–9.1)	0.19	1.4 (0.2–5.2)	0.40
PALB2	3 (0.43)	65 (0.12)	2 (0.40)	3.5 (0.7–10.3)	0.05	1.1 (0.2–3.1)	0.76
PMS2	2 (0.29)	56 (0.11)	1 (0.20)	2.7 (0.3–9.8)	0.17	1.4 (0.2–5.2)	0.40
RAD51C	1 (0.14)	59 (0.11)	2 (0.40)	1.3 (0.03–7.2)	0.54	0.4 (0.0–2.0)	0.54
RAD51D	3 (0.43)	40 (0.08)	1 (0.20)	5.7 (1.2–16.7)	0.02	2.2 (0.4–6.3)	0.16
XRCC2	0	23 (0.04)	0	—	—	—	—

* The denominators for genes for which data were censored were 561 (*BAP1*, *BARD1*, *BRIP1*, and *FAM175A*), 437 (*GEN1*), and 534 (*CHEK2*).
† Data are for the persons in the Exome Aggregation Consortium, minus the patients included in the TCGA studies. The percent with a mutation was calculated on the basis of the total number of persons for whom sequence coverage was adequate for the given allele, which differed slightly from the total of 53,105 persons, depending on the specific mutation.
‡ Data for metastatic cases with inadequate sequencing for this gene were censored.

(3 [4%]), and *PALB2* (3 [4%]) (Fig. 2). Four genes had no clearly detrimental aberrations. One man had mutations in *ATM* and *CHEK2*, and one man had mutations in *BRCA2* and *CHEK2*. The majority of men with DNA-repair gene mutations for whom the Gleason score was available (73 men) had primary tumors with high scores (Gleason scores range from 2 to 10, with higher scores associated with worse clinical outcomes): 56 men (77%) had a Gleason score of 8 through 10, 15 men (21%) had a score of 7, and 2 men (3%) had a score of 6. We found no association between the presence of a germline DNA-repair gene mutation and an age at diagnosis of younger than 60 years versus 60 years or older (P=0.90) or non-Hispanic white versus other race (P=0.84). There was marginal evidence that the presence of a germline DNA-repair gene mutation was associated with a Gleason score of 8 through 10 versus 7 or lower (odds ratio, 1.8; 95% confidence interval [CI], 1.0 to 3.5; P=0.04).

FAMILY CANCER HISTORY

Information regarding family history was available for 72 of 82 men (88%) with presumed pathogenic mutations in DNA-repair genes and for 537 of 610 men (88%) without DNA-repair gene mutations. In both groups, 22% of the men (16 of 72 men with DNA-repair gene mutations and 117 of 537 men without such mutations) had

The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

a first-degree relative with prostate cancer (P=1.0). However, 51 of the 72 patients with DNA-repair gene mutations (71%) had a first-degree relative with cancer other than prostate cancer, whereas 270 of the 537 patients without DNA-repair gene mutations (50%) had a first-degree relative with cancer other than prostate cancer (odds ratio, 2.4; 95% CI, 1.4 to 4.3; P=0.001). Inspection of extended pedigree information of probands with DNA-repair gene mutations revealed affected relatives with breast cancer (24 probands), ovarian cancer (10), leukemia and lymphoma (6), pancreatic cancer (7), or other gastrointestinal cancers (18).

Table 3. Germline DNA-Repair Gene Mutations in Seven Metastatic Prostate Cancer Case Series.

Case Series	Description	Patients	Patients with Mutations
		no.	*no. (%)*
1	Stand Up To Cancer–Prostate Cancer Foundation discovery series	150	15 (10.0)
2	Stand Up To Cancer–Prostate Cancer Foundation validation series	84	9 (10.7)
3	Royal Marsden Hospital	131	16 (12.2)
4	University of Washington	91	8 (8.8)
5	Weill Cornell Medical College	69	7 (10.1)
6	University of Michigan	43	4 (9.3)
7	Memorial Sloan Kettering Cancer Center	124	23 (18.5)
Total		692	82 (11.8)

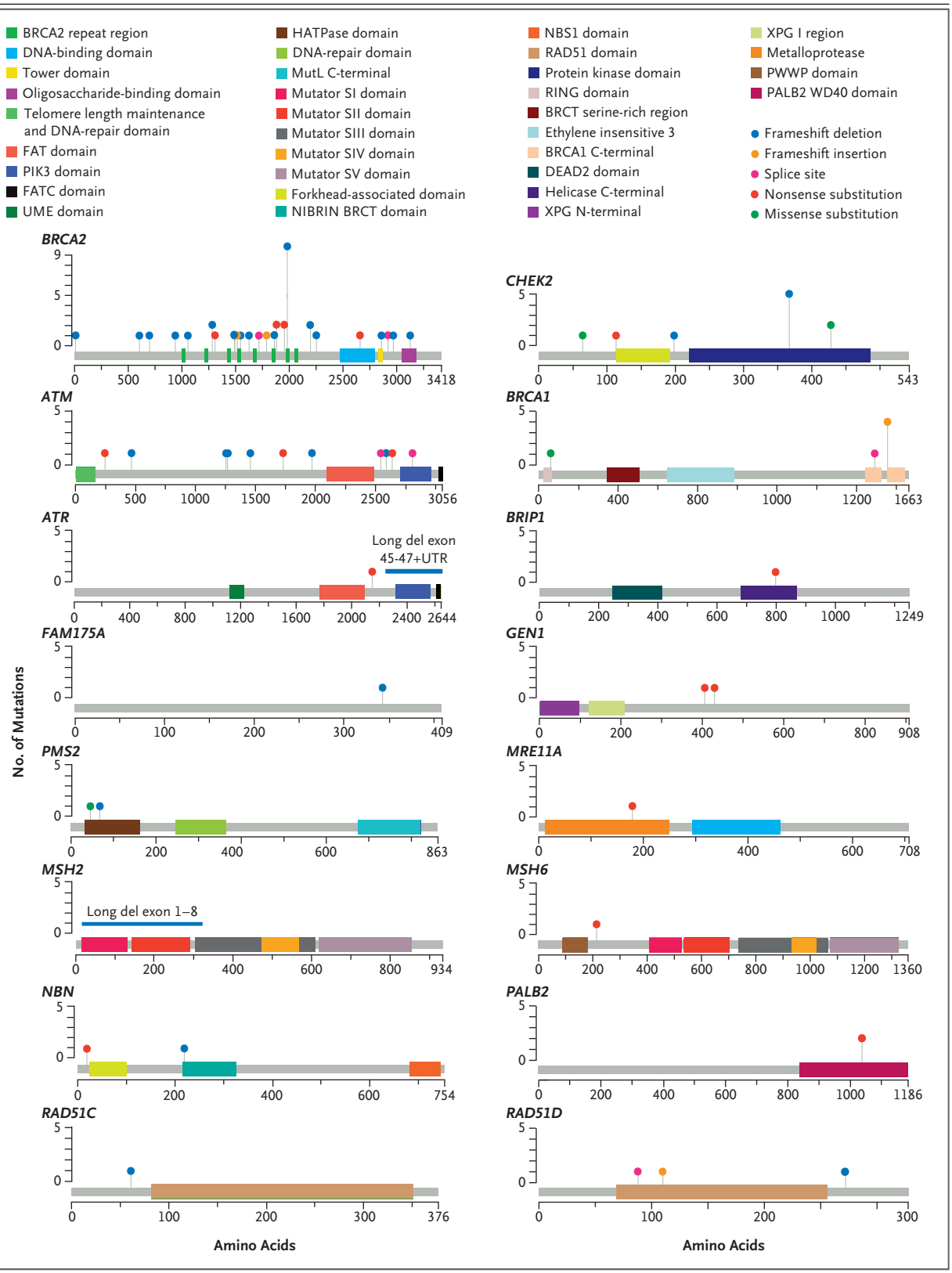
SOMATIC MUTATIONS IN DNA-REPAIR GENES

Tumor sequencing data were available for 61 of the men with germline DNA-repair gene mutations. For 36 (59%) of these men, the second allele was clearly aberrant, in that either a second loss-of-function mutation or a gene-copy loss was present (Table S1 in the Supplementary Appendix). A study of cancer-predisposition genes in children with cancer showed that 66% of children with a presumed pathogenic gene mutation had a second "hit" somatic aberration within the tumor genome,[28] and a study involving patients with advanced cancer showed that 21.4% of patients with a presumed pathogenic gene mutation had a somatic second-allele aberration.[23] Although a subset of germline loss-of-function mutations may not represent the causal event in the genesis of a given tumor, inactivation of the remaining allele may occur through epigenetic mechanisms or other processes.[29]

GERMLINE MUTATIONS IN DNA-REPAIR GENES IN LOCALIZED PROSTATE CARCINOMAS

We compared the frequency of germline DNA-repair gene mutations among men with metastatic prostate cancer with the frequency of such mutations among men with localized prostate cancer. In the Cancer Genome Atlas prostate cancer study,[25] which included 499 men for whom germline whole-exome sequencing data were available, 23 men (4.6%) had germline mutations in DNA-repair genes (P<0.001 for the comparison with metastatic disease). In addition, 6 men harbored the *BRCA2* K3326* polymorphism, a C-terminal truncating variant that is unlikely to be associated with a predisposition to prostate cancer.[30] It should be noted that to accommodate Cancer Genome Atlas requirements, the majority of tumors had high-risk characteristics: 90% were clinical stage T2c or greater, and 91% of the carcinomas had a Gleason score higher than 6, which far exceeds the approximately 30% of cancers with a Gleason score higher than 6 that was reported among men whose cancer was diagnosed by screening.[31-33] Presumed pathogenic mutations in DNA-repair genes were identified in 2 of 45 men (4%) who had cancer with a Gleason score of 6, in 9 of 249 men (4%) who had cancer with a Gleason score of 7, and in 12 of 205 men (6%) who had cancer with a Gleason score of 8, 9, or 10 (P=0.37 for trend). Four of 162 men (2%) with localized low-to-intermediate–risk tumors and 19 of 337 men (6%) with localized high-risk tumors, as categorized according to National Comprehensive Cancer Network risk criteria,[34] had germline DNA-repair gene mutations (Table 1). The odds of DNA-repair gene mutations being present among men with metastatic prostate cancer differed significantly from the odds among men with localized low-to-intermediate–risk tumors (odds ratio, 5.3; 95% CI, 1.9 to 20.2; P<0.001) or among those with high-risk tumors (odds ratio, 2.2; 95% CI, 1.3 to 4.0; P=0.002) (Table S6 in the Supplementary Appendix). As observed in men with metastatic prostate cancer, there was no association between the presence of a germline mutation in a DNA-repair gene and an age at diagnosis of younger than

The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.



The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

Figure 1 (facing page). Presumed Pathogenic Germline Mutations.

Locations of mutations and domains in proteins encoded by 16 predisposition genes are shown by lollipop structures, with the mutation type indicated by color. Protein domains are also distinguished by color. On the graph of each gene, the x axis reflects the number of amino acid residues, and the y axis represents the total number of mutations identified. Of the 20 genes analyzed, 4 (*BAP1*, *BARD1*, *MLH1*, and *XRCC2*) had no presumed pathogenic germline mutations.

60 versus 60 years of age or older (P=0.28) or non-Hispanic white versus other race (P=0.39).

GERMLINE MUTATIONS IN DNA-REPAIR GENES IN THE POPULATION

To estimate the population frequencies of germline mutations in DNA-repair genes, we analyzed exome data compiled from 53,105 persons included in the Exome Aggregation Consortium. We excluded data from persons with cancer who had been included in the Cancer Genome Atlas studies, the inclusion of which could have biased the comparisons with men with prostate cancer. The odds of any deleterious DNA-repair gene mutation being present in men with metastatic prostate cancer differed significantly from the odds in the Exome Aggregation Consortium population (odds ratio, 5.0; 95% CI, 3.9 to 6.3; P<0.001); a similar result was obtained when men from the previously reported Case Series 1 were excluded (odds ratio, 5.2; 95% CI, 4.0 to 6.8; P<0.001) (Table S5 in the Supplementary Appendix). The relative risk of mutations in individual DNA-repair genes among men with metastatic prostate cancer, as compared with men in the Exome Aggregation Consortium population, was substantial, ranging from 18.6 (95% CI, 13.2 to 25.3; P<0.001) for *BRCA2* to 3.1 (95% CI, 1.5 to 5.6; P=0.002) for *CHEK2* (Table 2).

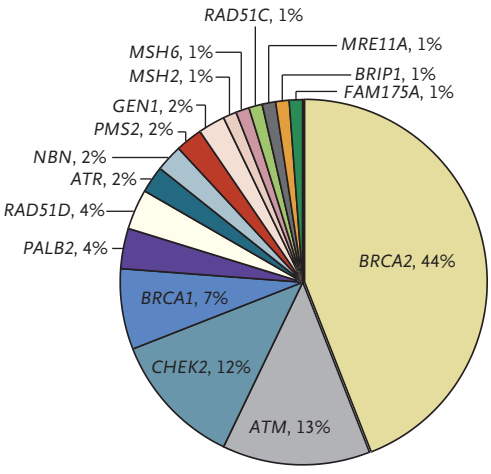


Figure 2. Distribution of Presumed Pathogenic Germline Mutations.

Shown are mutations involving 16 DNA-repair genes. Four genes did not have any pathogenic mutations identified and are not included in the distribution.

DISCUSSION

Inherited and acquired defects in DNA damage repair are key mechanisms in the genesis of malignant tumors. The detection of mutations in DNA-repair genes identifies persons and families who have a predisposition to cancer and defines cancer subtypes that have distinct vulnerabilities to specific therapeutics.[35] The ascertainment of germline mutations in DNA-repair genes in men with prostate cancer has several important clinical implications. First, the recent finding that pharmacologic inhibitors of PARP1 induce substantial objective responses in patients with metastatic prostate cancer expressing homologous recombination DNA-repair defects provides a clear treatment pathway in accordance with precision medicine strategies.[16] These tumors also appear to be responsive to platinum-based chemotherapy,[17] as has been documented for cancers of the ovary and breast in carriers of *BRCA1* and *BRCA2* mutations.[36,37] Second, the identification of a germline mutation in a DNA-repair gene provides information that is key to relatives, both male and female, and that can prompt "cascade" counseling to identify cancer predisposition and deploy risk-reduction strategies. Prospective studies assessing the prognostic and predictive significance of mutations in DNA-repair genes with regard to clinical outcomes are now needed to inform personalized care.

The significant family history of nonprostate cancers among men with mutations in DNA-repair genes was largely accounted for by breast, ovarian, and pancreatic cancers, in which mutations in DNA-repair pathways are known. The possible association between mutations in DNA-repair genes and familial hematologic and gas-

The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

trointestinal cancers requires further analysis of cosegregation in affected kindreds. As observed for *BRCA1* and *BRCA2* in breast cancer, mutations may be found in persons who do not have a known syndromic history.[38,39] Thus, broader testing of patients with metastatic prostate cancer without regard to family history will increase the yield of actionable mutations identified, in a manner parallel to the recent inclusion of all patients with epithelial ovarian cancers for germline testing regardless of family history.[40]

This study has several limitations. First, although efforts were made to standardize DNA-sequencing analyses, direct comparability across institutions and with public data is not guaranteed. Second, we focused on clearly deleterious mutations in a selected set of DNA-repair genes; consequently, our findings may underestimate the true frequency of pathogenic events that influence the development of metastatic prostate cancer. Third, although patients across institutions and in the control populations were unselected for family history, possible bias cannot be ruled out. Finally, our case series and the Cancer Genome Atlas study include few persons who were older than 70 years of age at diagnosis, and the incidence of germline DNA-repair gene mutations may differ in this older age group.

In conclusion, the 11.8% overall frequency of germline aberrations in genes responsible for maintaining DNA integrity in men with metastatic prostate cancer is substantially higher than the 1.2 to 1.8% incidence of *BRCA2* mutations alone in localized prostate cancer[9,12] or the 7.3% incidence of mutations in 22 tumor-suppressor genes in familial prostate cancer.[14] Because the high frequency of DNA-repair gene mutations is not exclusive to an early-onset phenotype and is associated with clinically and histologically aggressive disease, with compelling evidence for therapeutic relevance, it may be of interest to routinely examine all men with metastatic prostate cancer for the presence of germline mutations in DNA-repair genes.

Supported by a Stand Up To Cancer–Prostate Cancer Foundation (SU2C-PCF) International Prostate Cancer Dream Team Translational Cancer Research Grant. Stand Up To Cancer is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research (SU2C-AACR-DT0712). The project was also supported by the following National Institutes of Health and Department of Defense awards: Prostate SPORE (grants P50CA097186 and P50CA92629, P30CA008748, PC131820, PC140799, R01CA116337, and 1K08CA188615) and the Prostate Cancer Foundation (Movember Challenge Awards). Drs. Beltran and Van Allen are supported by Damon Runyon Clinical Investigator Awards. Drs. Pritchard, Abida, Cheng, Schultz, and Van Allen are supported by Prostate Cancer Foundation Young Investigator Awards. Drs. Chinnaiyan and Sawyers are supported by the Howard Hughes Medical Research Institute. We also acknowledge funding from the Richard M. Lucas Foundation, the Institute for Prostate Cancer Research, Prostate Cancer UK and Movember to the London Prostate Cancer Centre of Excellence, the Starr Cancer Consortium (support to Drs. Beltran and Rubin), a Medical Research Council-Prostate Cancer UK Fellowship (to Dr. Mateo), an Experimental Cancer Medical Centre grant, a Biomedical Research Centre grant to the Institute of Cancer Research–Royal Marsden, the Andrew Sabin Family Foundation, the Marie-Josée and Henry R. Kravis Center for Molecular Oncology, and the Robert and Kate Niehaus Center for Inherited Cancer Genomics at Memorial Sloan Kettering Cancer Center.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank the men who participated in this study for helping us to gain a better understand the role of genetic predisposition in advanced prostate cancer, the investigators and staff participating in the Stand Up to Cancer–Prostate Cancer Foundation International Prostate Cancer Dream Team, and the following persons at our respective institutions who helped with this study: Hiep Nguyen, Mary-Claire King, Barbara Norquist, Celestia Higano, Lawrence True, and Robert Vessella (University of Washington); Claudia Bertan, Susana Miranda, Penny Flohr, Roberta Ferraldeschi, Zsofia Kote-Jarai, Bindu Raobaikady, Ajit Sarvadikar, Dionne Alleyne, Lucy Hamilton, Sheena Vadgama, and Ada Balasopoulou (Institute for Cancer Research); Jacob Musinsky, Josh Armenia, Diana Mandelker, Maria Arcila, and David Hyman (Memorial Sloan Kettering Cancer Center); Xuhong Cao, Yi-Mi Wu, and Felix Feng (University of Michigan); Elizabeth Heath (Wayne State University); and Tuo Zhang (Weill Cornell Medical College). We also thank the Exome Aggregation Consortium and the groups that provided exome variant data for comparison; a full list of groups contributing to the Exome Aggregation Consortium can be found at http://exac.broadinstitute.org/about.

APPENDIX

The authors' full names and academic degrees are as follows: Colin C. Pritchard, M.D., Ph.D., Joaquin Mateo, M.D., Michael F. Walsh, M.D., Navonil De Sarkar, Ph.D., Wassim Abida, M.D., Ph.D., Himisha Beltran, M.D., Andrea Garofalo, B.Sc., Roman Gulati, M.Sc., Suzanne Carreira, Ph.D., Rosalind Eeles, M.D., Ph.D., Olivier Elemento, Ph.D., Mark A. Rubin, M.D., Dan Robinson, Ph.D., Robert Lonigro, Ph.D., Maha Hussain, M.D., Arul Chinnaiyan, M.D., Ph.D., Jake Vinson, B.Sc., Julie Filipenko, M.Sc., Levi Garraway, M.D., Ph.D., Mary-Ellen Taplin, M.D., Saud AlDubayan, M.D., G. Celine Han, Ph.D., Mallory Beightol, B.Sc., Colm Morrissey, Ph.D., Belinda Nghiem, B.Sc., Heather H. Cheng, M.D., Ph.D., Bruce Montgomery, M.D., Tom Walsh, Ph.D., Silvia Casadei, Ph.D., Michael Berger, Ph.D., Liying Zhang, M.D., Ph.D., Ahmet Zehir, Ph.D., Joseph Vijai, Ph.D., Howard I. Scher, M.D., Charles Sawyers, M.D., Nikolaus Schultz, Ph.D., Philip W. Kantoff, M.D., David Solit, M.D., Mark Robson, M.D., Eliezer M. Van Allen, M.D., Kenneth Offit, M.D., Johann de Bono, M.B., Ch.B., Ph.D., and Peter S. Nelson, M.D.

The authors' affiliations are as follows: the University of Washington (C.C.P., M. Beightol, C.M., B.N., H.H.C., B.M., T.W., S. Casadei, P.S.N.) and Fred Hutchinson Cancer Research Center (N.D.S., R.G., P.S.N.) — both in Seattle; the Institute of Cancer Research and Royal Marsden Hospital, London (J.M., S. Carreira, R.E., J.B.); Memorial Sloan Kettering Cancer Center (M.F.W., W.A., M. Berger, L.Z., A.Z.,

The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

J. Vijai, H.I.S., C.S., N.S., P.W.K., D.S., M.R., K.O.), Weill Cornell Medical College (H.B., O.E., M.A.R.), and the Prostate Cancer Clinical Trials Consortium (J. Vinson, J.F.) — all in New York; the University of Michigan, Ann Arbor (D.R., R.L., M.H., A.C.); Howard Hughes Medical Institute, Chevy Chase, MD (A.C., C.S.); and Dana–Farber Cancer Institute, Boston (A.G., L.G., M.-E.T., S.A., G.C.H., E.M.V.A.).

REFERENCES

1. Albertsen PC, Hanley JA, Fine J. 20-Year outcomes following conservative management of clinically localized prostate cancer. JAMA 2005;293:2095-101.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66:7-30.
3. Mucci LA, Hjelmborg JB, Harris JR, et al. Familial risk and heritability of cancer among twins in Nordic countries. JAMA 2016;315:68-76.
4. Amin Al Olama A, Kote-Jarai Z, Schumacher FR, et al. A meta-analysis of genome-wide association studies to identify prostate cancer susceptibility loci associated with aggressive and non-aggressive disease. Hum Mol Genet 2013;22:408-15.
5. Szulkin R, Karlsson R, Whitington T, et al. Genome-wide association study of prostate cancer-specific survival. Cancer Epidemiol Biomarkers Prev 2015;24:1796-800.
6. Helfand BT, Roehl KA, Cooper PR, et al. Associations of prostate cancer risk variants with disease aggressiveness: results of the NCI-SPORE Genetics Working Group analysis of 18,343 cases. Hum Genet 2015;134:439-50.
7. Berndt SI, Wang Z, Yeager M, et al. Two susceptibility loci identified for prostate cancer aggressiveness. Nat Commun 2015;6:6889.
8. Haraldsdottir S, Hampel H, Wei L, et al. Prostate cancer incidence in males with Lynch syndrome. Genet Med 2014;16:553-7.
9. Kote-Jarai Z, Leongamornlert D, Saunders E, et al. BRCA2 is a moderate penetrance gene contributing to young-onset prostate cancer: implications for genetic testing in prostate cancer patients. Br J Cancer 2011;105:1230-4.
10. Leongamornlert D, Mahmud N, Tymrakiewicz M, et al. Germline BRCA1 mutations increase prostate cancer risk. Br J Cancer 2012;106:1697-701.
11. Xu J, Lange EM, Lu L, et al. HOXB13 is a susceptibility gene for prostate cancer: results from the International Consortium for Prostate Cancer Genetics (ICPCG). Hum Genet 2013;132:5-14.
12. Gallagher DJ, Gaudet MM, Pal P, et al. Germline BRCA mutations denote a clinicopathologic subset of prostate cancer. Clin Cancer Res 2010;16:2115-21.
13. Castro E, Goh C, Olmos D, et al. Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer. J Clin Oncol 2013;31:1748-57.
14. Leongamornlert D, Saunders E, Dadaev T, et al. Frequent germline deleterious mutations in DNA repair genes in familial prostate cancer cases are associated with advanced disease. Br J Cancer 2014;110:1663-72.
15. Loeb S, Bjurlin MA, Nicholson J, et al. Overdiagnosis and overtreatment of prostate cancer. Eur Urol 2014;65:1046-55.
16. Mateo J, Carreira S, Sandhu S, et al. DNA-repair defects and olaparib in metastatic prostate cancer. N Engl J Med 2015;373:1697-708.
17. Cheng HH, Pritchard CC, Boyd T, Nelson PS, Montgomery B. Biallelic inactivation of BRCA2 in platinum-sensitive metastatic castration-resistant prostate cancer. Eur Urol 2016;69:992-5.
18. Robinson D, Van Allen EM, Wu YM, et al. Integrative clinical genomics of advanced prostate cancer. Cell 2015;161:1215-28.
19. Pritchard CC, Morrissey C, Kumar A, et al. Complex MSH2 and MSH6 mutations in hypermutated microsatellite unstable advanced prostate cancer. Nat Commun 2014;5:4988.
20. Pritchard CC, Smith C, Salipante SJ, et al. ColoSeq provides comprehensive Lynch and polyposis syndrome mutational analysis using massively parallel sequencing. J Mol Diagn 2012;14:357-66.
21. Pritchard CC, Salipante SJ, Koehler K, et al. Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. J Mol Diagn 2014;16:56-67.
22. Beltran H, Eng K, Mosquera JM, et al. Whole-exome sequencing of metastatic cancer and biomarkers of treatment response. JAMA Oncol 2015;1:466-74.
23. Schrader KA, Cheng DT, Joseph V, et al. Germline variants in targeted tumor sequencing using matched normal DNA. JAMA Oncol 2016;2:104-11.
24. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-24.
25. Cancer Genome Atlas Research Network. The molecular taxonomy of primary prostate cancer. Cell 2015;163:1011-25.
26. Shirts BH, Casadei S, Jacobson AL, et al. Improving performance of multigene panels for genomic analysis of cancer predisposition. Genet Med 2016 February 4 (Epub ahead of print).
27. Walsh T, Lee MK, Casadei S, et al. Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing. Proc Natl Acad Sci USA 2010;107:12629-33.
28. Zhang J, Walsh MF, Wu G, et al. Germline mutations in predisposition genes in pediatric cancer. N Engl J Med 2015;373:2336-46.
29. Tapia T, Smalley SV, Kohen P, et al. Promoter hypermethylation of BRCA1 correlates with absence of expression in hereditary breast cancer tumors. Epigenetics 2008;3:157-63.
30. Meeks HD, Song H, Michailidou K, et al. BRCA2 polymorphic stop codon K3326X and the risk of breast, prostate, and ovarian cancers. J Natl Cancer Inst 2016;108:108.
31. Wilt TJ, Brawer MK, Jones KM, et al. Radical prostatectomy versus observation for localized prostate cancer. N Engl J Med 2012;367:203-13.
32. Schröder FH, Hugosson J, Roobol MJ, et al. Screening and prostate-cancer mortality in a randomized European study. N Engl J Med 2009;360:1320-8.
33. Schröder FH, Hugosson J, Carlsson S, et al. Screening for prostate cancer decreases the risk of developing metastatic disease: findings from the European Randomized Study of Screening for Prostate Cancer (ERSPC). Eur Urol 2012;62:745-52.
34. Mohler JL, Armstrong AJ, Bahnson RR, et al. Prostate cancer, version 1.2016. J Natl Compr Canc Netw 2016;14:19-30.
35. Jeggo PA, Pearl LH, Carr AM. DNA repair, genome stability and cancer: a historical perspective. Nat Rev Cancer 2016;16:35-42.
36. Isakoff SJ, Mayer EL, He L, et al. TBCRC009: a multicenter phase II clinical trial of platinum monotherapy with biomarker assessment in metastatic triple-negative breast cancer. J Clin Oncol 2015;33:1902-9.
37. Alsop K, Fereday S, Meldrum C, et al. BRCA mutation frequency and patterns of treatment response in BRCA mutation-positive women with ovarian cancer: a report from the Australian Ovarian Cancer Study Group. J Clin Oncol 2012;30:2654-63.
38. King MC, Marks JH, Mandell JB. Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. Science 2003;302:643-6.
39. Weitzel JN, Lagos VI, Cullinane CA, et al. Limited family structure and BRCA gene mutation status in single cases of breast cancer. JAMA 2007;297:2587-95.
40. National Comprehensive Cancer Network. Genetic/familial high-risk assessment: breast and ovarian, version 2.2016. (https://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf).

Copyright © 2016 Massachusetts Medical Society.

The New England Journal of Medicine
Downloaded from nejm.org by Colin Pritchard on July 6, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

nature medicine

Classification and characterization of microsatellite instability across 18 cancer types

Ronald J Hause[1], Colin C Pritchard[2], Jay Shendure[1,3] & Stephen J Salipante[2]

Microsatellite instability (MSI), the spontaneous loss or gain of nucleotides from repetitive DNA tracts, is a diagnostic phenotype for gastrointestinal, endometrial, and colorectal tumors, yet the landscape of instability events across a wider variety of cancer types remains poorly understood. To explore MSI across malignancies, we examined 5,930 cancer exomes from 18 cancer types at more than 200,000 microsatellite loci and constructed a genomic classifier for MSI. We identified MSI-positive tumors in 14 of the 18 cancer types. We also identified loci that were more likely to be unstable in particular cancer types, resulting in specific instability signatures that involved cancer-associated genes, suggesting that instability patterns reflect selective pressures and can potentially identify novel cancer drivers. We also observed a correlation between survival outcomes and the overall burden of unstable microsatellites, suggesting that MSI may be a continuous, rather than discrete, phenotype that is informative across cancer types. These analyses offer insight into conserved and cancer-specific properties of MSI and reveal opportunities for improved methods of clinical MSI diagnosis and cancer gene discovery.

© 2016 Nature America, Inc. All rights reserved.

MSI is a molecular tumor phenotype resulting from genomic hypermutability. The gain or loss of nucleotides from microsatellite tracts—DNA elements composed of short repeating motifs—is the diagnostic hallmark of MSI[1] and manifests as novel alleles of varying length[2]. These changes can arise from impairments in the mismatch repair (MMR) system, which limits correction of spontaneous mutations in repetitive DNA sequences[3,4]. MSI-affected tumors may, accordingly, result from mutational inactivation or epigenetic silencing of genes in the MMR pathway[2,3]. MSI is classically associated with colorectal cancers, for which it holds well-defined clinical implications[3]. However, MSI has been reported in diverse cancer types including endometrial, ovarian, gastric, and prostate cancer and glioblastoma[3,5,6]. Recent work suggests that MSI may be an actionable marker for immune-checkpoint-blockade therapy; clinical trials have demonstrated improved outcomes for patients with MSI-positive tumors treated with inhibitors of programmed cell death 1 (PD-1), presumably as a result of T lymphocyte recognition of neoantigens produced by somatic mutations[7,8]. However, mutations resulting from MSI can also drive oncogenesis, by inactivating tumor suppressor genes, for example[9]. These observations underscore the need for a more complete understanding of MSI.

MSI signatures may differ among cancer types; disparate loci may be preferentially unstable[5,10–14], MSI positivity may carry different prognostic values[11], and MSI may occur at different frequencies[5] across malignancies. However, these observations come from examination of dozens of loci in cohorts no larger than 100 individuals. Beyond limited studies restricted to four cancer types with established MSI phenotypes[10,15,16], variation in MSI among malignancies has not yet been evaluated systematically or on a genomic scale.

Molecular diagnosis of MSI is currently achieved by examining PCR products from a few (typically 5–7) informative microsatellite markers (MSI–PCR)[1,17]. Recently, our group and others[10,18–22] developed methods to infer MSI using massively parallel DNA-sequencing technologies, enabling interrogation of MSI with a breadth and quantitative precision not previously achievable. Here, we describe a robust approach for predicting MSI status independently of cancer type and use tumor exomes from the Cancer Genome Atlas (TCGA) Research Network to more comprehensively examine MSI across tumor types.

RESULTS

MSI classifier

From a total of 19,075,236 microsatellites computationally identified across the human genome, we included a subset of 516,876 loci (2.7%) that were within or adjacent to the exome capture baits used by TCGA, representing 95.9% of all coding microsatellites and 98.4% of microsatellites occupying splice sites (**Fig. 1a**,**b** and **Supplementary Table 1**). These loci were primarily mononucleotide repeats (**Supplementary Table 2**) and, as expected from our study design, fell disproportionately into intronic and coding regions compared to distributions observed genome-wide (**Fig. 1b** and **Supplementary Table 3**). Insufficient sequencing read depth precluded interrogation of all microsatellites for every specimen: 223,082 loci (43%) had sufficient coverage (≥30 reads) in both tumor and normal tissue for instability status to be inferred in at least half of the 5,930 total specimens.

For each locus we catalogued microsatellite allele lengths in tumor and patient-matched normal exomes (**Fig. 1c**, **Supplementary Fig. 1**, and **Supplementary Tables 4** and **5**) to identify and quantify MSI events. Using these instability calls, we designed a classifier to

[1]Department of Genome Sciences, University of Washington, Seattle, Washington, USA. [2]Department of Laboratory Medicine, University of Washington, Seattle, Washington, USA. [3]Howard Hughes Medical Institute, Seattle, Washington, USA. Correspondence should be addressed to S.J.S. (stevesal@uw.edu) or J.S. (shendure@uw.edu).

Received 3 May; accepted 29 August; published online 3 October 2016; doi:10.1038/nm.4191

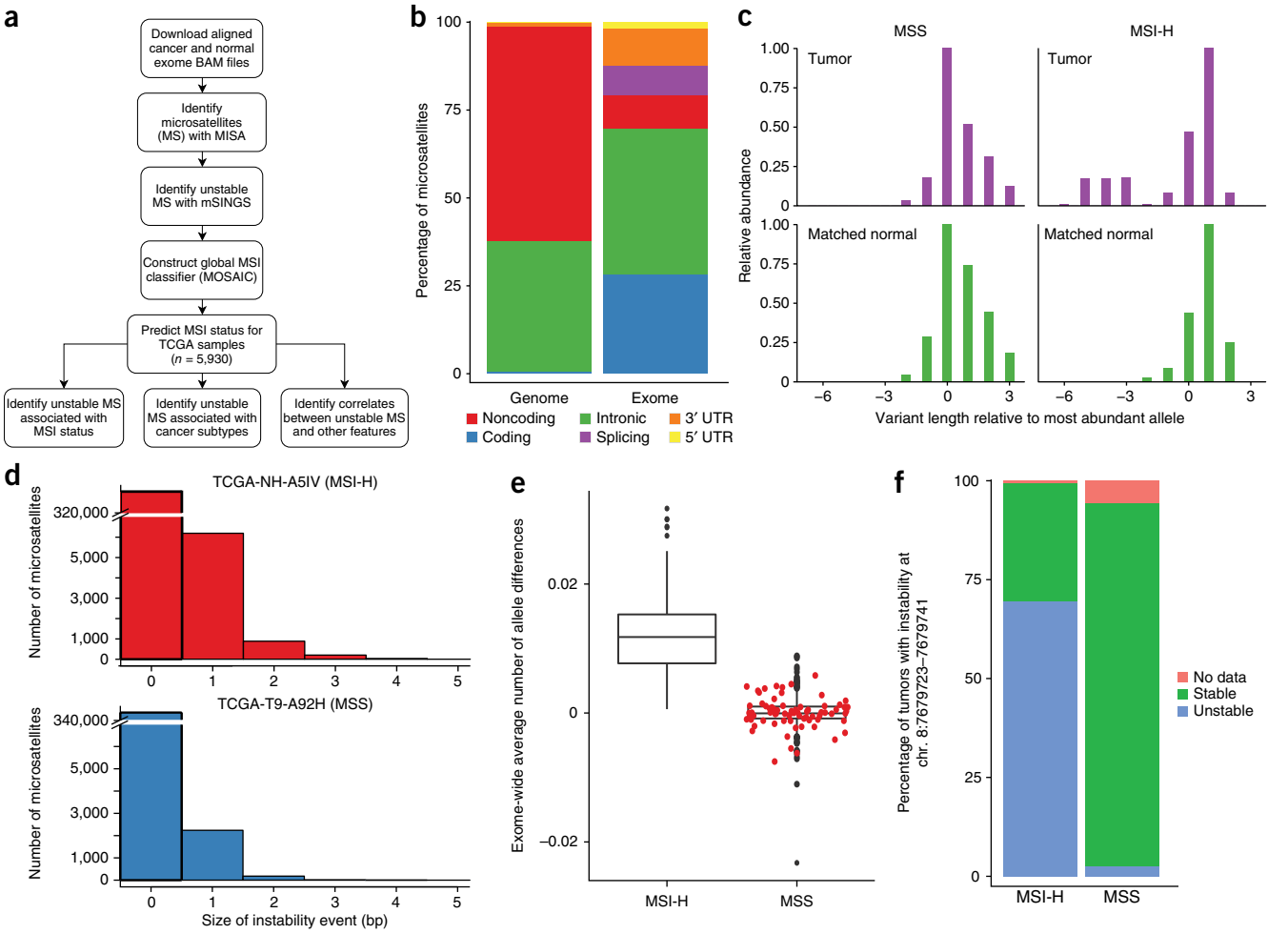


Figure 1 Evaluating MSI using exome-sequencing data. (**a**) Schematic of the approach used for analyzing MSI across TCGA exomes. MISA, microsatellite identification tool, mSINGS, microsatellite instability by next-generation sequencing. (**b**) Relative proportions of microsatellite loci within indicated genomic annotations across the whole genome and regions targeted by exome capture. Data represent computational identification and annotation of all microsatellites in the human reference genome and the subset within or immediately adjacent to TCGA exome capture baits. (**c**) Detection of MSI events from sequencing data. Representative virtual electropherograms[21] of a compound repeat at chr. 1:33145935–33145982 are illustrated for MSS and MSI-H cases, comparing the length and relative abundance of microsatellite alleles between tumors and patient-matched normal material. (**d**) Size of MSI events in representative MSI-H and MSS colon cancers. TCGA patient identifiers are indicated. (**e**) Correlation between MSI status (diagnosed using conventional clinical methods; MSI-H $n = 171$, MSS $n = 446$) and differences in global measurements of locus instability in tumor and paired normal specimens. Box boundaries indicate the interquartile range; center lines, medians; whiskers, values within 1.5 interquartile ranges of median; circles, extreme outliers. Red points represent MSI-L cancers ($n = 73$). (**f**) Proportion of MSI-H and MSS tumors with instability in a microsatellite locus located at chr. 8:7679723–7679741, within *DEFB105A/B*. This locus was the most significantly unstable microsatellite in MSI-H ($n = 171$) relative to MSS tumors ($n = 446$, $P = 2 \times 10^{-61}$, Fisher's exact test).

distinguish MSI-positive (MSI-high (MSI-H)) from MSI-negative (MSI-stable (MSS)) specimens independently of cancer type. Of all covariates tested across a cohort of colon, rectal, endometrial, and gastric tumors with available MSI–PCR results, the average total gain in the number of microsatellite alleles observed in a tumor relative to normal tissue across all microsatellite loci was the most significant feature separating MSI-H from MSS cancers (**Fig. 1d**,**e**; MSI-H median = 0.012, MSS median = -5.4×10^{-5}, $P = 9.4 \times 10^{-80}$, two-sided Wilcoxon rank-sum test). Related metrics, including the overall numbers of unstable microsatellites and variances in the allele number gain between tumor and normal, were also significantly different between MSI status groups (**Supplementary Fig. 2**; $P < 10^{-72}$, two-sided Wilcoxon rank-sum test). We also tested all microsatellite loci for discriminatory power to differentiate MSI-H from MSS samples and identified a locus within *DEFB105A* or *DEFB105B* (*DEFB105A/B*), chr. 8:7679723–7679741, as the most significantly unstable microsatellite in MSI-H tumors, as compared to MSS tumors (**Fig. 1f**; unstable in 119 of 171 MSI-H and 11 of 446 of MSS tumors, $P = 2 \times 10^{-61}$, two-sided Fisher's exact test). On the basis of these data, we created a parsimonious, weighted-tree microsatellite instability classifier (MOSAIC) for predicting MSI status using the most informative and independent features for classifying MSI—average gain of novel microsatellite alleles detected in a tumor specimen and, secondarily, locus instability within *DEFB105A/B* (**Supplementary Fig. 3a**). Incorporating additional covariates did not substantially improve the classifier (**Supplementary Fig. 3b**), nor did more sophisticated machine learning approaches. Compared with MSI–PCR, MOSAIC classified MSI-H from MSS cancers with 96.6% leave-one-sample-out cross-validation accuracy (95.8% sensitivity, 97.6% specificity) in a set of 617 specimens (128 MSS and 44 MSI-H for

© 2016 Nature America, Inc. All rights reserved.

colon; 63 MSS and 3 MSI-H for rectal; 169 MSS and 92 MSI-H for endometrial; 86 MSS and 32 MSI-H for stomach cancers). MOSAIC was discordant with clinical testing in classifying 11 of 171 MSI-H tumors (1 rectal and 10 endometrial) as MSS and 7 (1 rectal, 1 colon, and 5 endometrial) of 446 MSS cancers as MSI-H (**Supplementary Table 6**). Discordant classifications were primarily in endometrial cancers, which showed the smallest differences between MSI-H and MSS groups for all instability metrics measured. However, evidence suggests that many of these specimens were improperly classified by MSI–PCR: a review of accessory genetic and epigenetic data for somatic disruption of MSI-causative genes revealed that 7 of the 16 cases with complete metadata available were compatible with MOSAIC classifications but not MSI–PCR results (**Supplementary Table 6**). Furthermore, in terms of average number of gained microsatellite alleles and global burden of unstable microsatellites, discordant specimens were more consistent with MOSAIC classifications than with MSI–PCR testing (**Supplementary Fig. 4**).

Last, we evaluated whether differences in sequencing read depth between matched tumor and normal exomes or across microsatellite loci could confound our analysis. We observed no meaningful correlation between instability calls and these read depth metrics ($R^2 = 0.01$ and $\rho = -0.04$, respectively; **Supplementary Fig. 5**). Overall, these results demonstrate that we can accurately classify MSI status from tumor and matched-normal tissue exome-sequencing data.

Investigation of MSI-low phenotype

MSI-low (MSI-L) is a subcategory of MSI marked by instability at a minimal fraction of typed microsatellite markers. It is debated

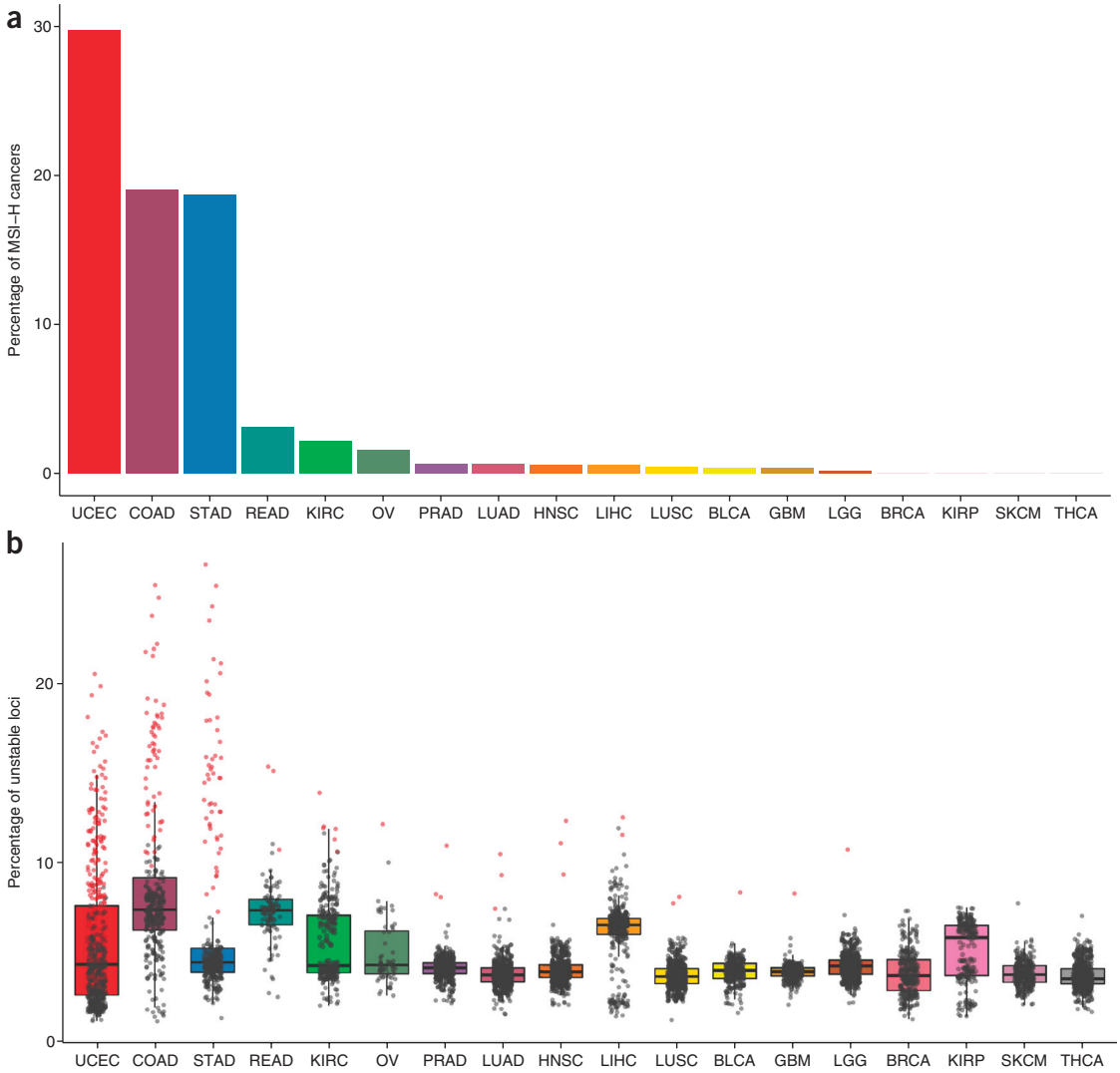


Figure 2 The landscape of MSI across TCGA exomes. (**a**) Inferred proportion of MSI-H tumors identified for each cancer cohort. (**b**) Distributions of the overall percentages of unstable microsatellite loci identified for each cancer type. Box boundaries indicate the interquartile range; center lines, medians; whiskers, values within 1.5 interquartile ranges of median. Overlaid points represent the number of unstable loci detected in individual tumor specimens; data for tumors classified as MSI-H are shown in red. UCEC, uterine corpus endometrial carcinoma ($n = 437$); COAD, colon adenocarcinoma ($n = 294$); STAD, stomach adenocarcinoma ($n = 278$); READ, rectal adenocarcinoma ($n = 96$); KIRC, kidney renal clear cell carcinoma ($n = 279$); OV, ovarian serous cystadenocarcinoma ($n = 63$); PRAD, prostate adenocarcinoma ($n = 463$); LUAD, lung adenocarcinoma ($n = 480$); HNSC, head and neck squamous cell carcinoma ($n = 506$); LIHC, liver hepatocellular carcinoma ($n = 338$); LUSC, lung squamous cell carcinoma ($n = 443$); BLCA, bladder urothelial carcinoma ($n = 253$); GBM, glioblastoma multiforme ($n = 262$); LGG, brain lower grade glioma ($n = 513$); BRCA, breast invasive carcinoma ($n = 266$); KIRP, kidney renal papillary cell carcinoma ($n = 207$); SKCM, skin cutaneous melanoma ($n = 268$); THCA, thyroid carcinoma ($n = 484$).

© 2016 Nature America, Inc. All rights reserved.

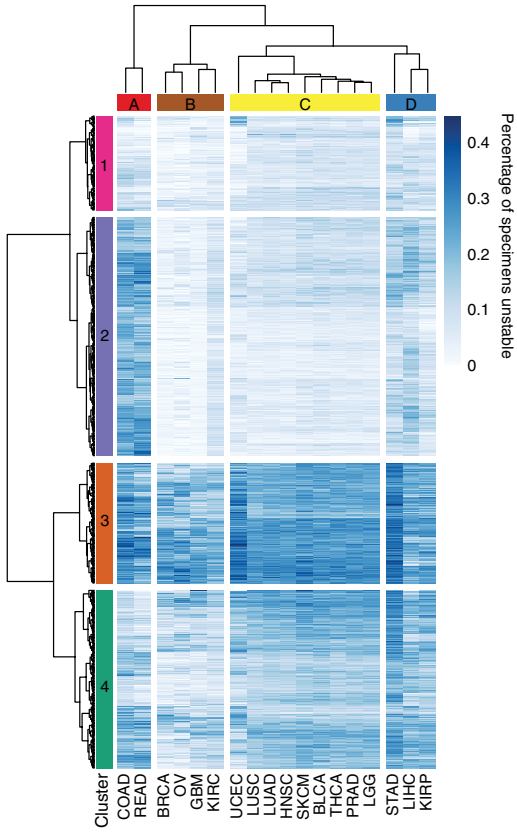


Figure 3 Cancer-specific signatures of MSI. Heatmap indicating the proportions of specimens within cancer types (columns) that were unstable at individual loci microsatellites (rows). Loci significant for differences among cancer types at FDR < 0.05 are shown. Colored microsatellite clusters (1–4, at left) denote groups of loci with similar instability trends based on Bayesian information criterion of the most likely model and number of clusters. Cancer types were also organized by hierarchical clustering into groups with similar patterns of MSI (A–D, top). UCEC, uterine corpus endometrial carcinoma; COAD, colon adenocarcinoma; STAD, stomach adenocarcinoma; READ, rectal adenocarcinoma; KIRC, kidney renal clear cell carcinoma; OV, ovarian serous cystadenocarcinoma; PRAD, prostate adenocarcinoma; LUAD, lung adenocarcinoma; HNSC, head and neck squamous cell carcinoma; LIHC, liver hepatocellular carcinoma; LUSC, lung squamous cell carcinoma; BLCA, bladder urothelial carcinoma; GBM, glioblastoma multiforme; LGG, brain lower grade glioma; BRCA, breast invasive carcinoma; KIRP, kidney renal papillary cell carcinoma; SKCM, skin cutaneous melanoma; THCA, thyroid carcinoma.

whether MSI-L is a distinct disease entity or an artifact of examining small numbers of loci through conventional MSI testing[23]. We therefore examined exome instability covariates in colon, rectal, endometrial, and stomach cancers clinically categorized as MSI-L. We observed no significant differences between MSI-L and MSS cancers in numbers of gained microsatellite alleles in tumor relative to normal tissue (P = 0.73, two-sided Wilcoxon rank-sum test), overall variation in allele number differences across all loci (P = 0.10), or total number of unstable microsatellites (P = 0.20; **Fig. 1f** and **Supplementary Fig. 2**). The lack of observable differences between these categories supports previous observations[10] and indicates that MSI-L tumors are consistent with MSS tumors in overall MSI burden. We reclassified MSI-L tumors as MSS for all subsequent analyses.

MSI status and landscape across different cancers

We broadly applied MOSAIC to assign MSI status for 5,930 tumor exomes from 18 cancer types (**Fig. 2a** and **Supplementary Tables 4** and **5**), enabling us to extend the analysis to 15 additional cancer types for which MSI status is not tested in clinical practice and to identify an additional 93 MSI-H samples. Cancer exomes contained a wide range of unstable microsatellites, from 87 to 9,032 (**Supplementary Table 5**). The average number of unstable sites varied considerably by cancer type, from a minimum of 765, for thyroid carcinomas, to a maximum of 2,315, for colon cancers. Similarly, the fraction of inferred MSI-H tumors also varied. The highest proportion of MSI-H cases occurred in cancer types that classically demonstrate MSI: endometrial (30%), colon (19%), and gastric (19%). Rectal cancers had a lower prevalence of MSI-H specimens (3%). Still lower, but detectable, frequencies of MSI-H were observed in 12 other cancer types; collectively, one or more individual MSI-H tumors were identified in 16 of the 18 cancer types examined. For several cancer types, including kidney papillary, kidney clear cell, and liver hepatocellular carcinomas, we observed a bimodal distribution in the proportion of unstable microsatellites for cancers classified as MSS (**Fig. 2b**), indicating trends in instability rates within MSI classifications.

As anticipated, we observed a strong correlation between predicted MSI status and the occurrence of somatic mutations or epigenetic silencing in MMR-pathway and DNA proofreading genes (odds ratio (OR) = 13.7 for having a somatic mutation in MSI-H malignancies compared with MSS, $P = 6 \times 10^{-64}$; **Supplementary Table 7**). Notably, these somatic alterations did not predict MSI-H status with high accuracy, suggesting contributions of additional factors to MSI. Despite the well-established role of mismatch repair gene *MLH1* silencing in MSI-H tumors[1], 8 of 98 tumors with *MLH1* silencing were classified as MSS by both MOSAIC and MSI–PCR.

To provide a more comprehensive view of the MSI landscape within and across cancer types, we next examined global patterns of microsatellite mutation using instability calls for individual loci. We included all specimens, irrespective of inferred MSI status, and restricted analysis to 92,385 microsatellites that were called in at least half of the samples across each of the 18 cancer types (**Supplementary Table 8**). No instability was observed at 57.4% of loci in any tumor. Of the sites that were unstable in at least 5% of specimens, hierarchical clustering distinguished four major groups (A–D) of cancer types having similar signatures of MSI (**Fig. 3**). Cancers that are canonically affected by MSI were distributed between three different groups: colon and rectal cancers exclusively comprised group A, whereas stomach cancers were placed in a separate category with liver hepatocellular and kidney renal carcinoma (D), and endometrial tumors were separately grouped with multiple other cancer types (C). Other malignancies, representing those with lower or no inferred incidences of MSI-H, were distributed among three groups (B, C, and D) but were disproportionately allocated to group C. All cancer types, including those entirely comprising MSS tumors, showed high frequencies of instability events at particular loci or groups of similarly mutated loci. The microsatellite loci were also partitioned by hierarchical clustering into four major divisions (1–4) that showed similar rates of instability across cancer groups (**Fig. 3**). We examined enrichment of gene ontologies and KEGG pathway annotations of factors harboring unstable microsatellites in each division and noted differences (**Supplementary Fig. 6** and **Supplementary Table 9**) but observed no obvious patterns of biological function.

© 2016 Nature America, Inc. All rights reserved.

Differences between MSS and MSI-H cancers

Because MSS tumors have a low baseline level of MSI[24], we examined whether MSS tumors mutate at the same loci as tissue-matched MSI-H tumors by comparing their relative frequencies of instability events at each microsatellite. For sufficient numbers, we focused on the four cancer types with the highest incidence of MSI-H samples (colon, rectal, endometrial, and stomach). Although both the frequency of instability events and the number of alternative microsatellite alleles were significantly elevated in MSI-H tumors, they tended to occur at the same loci that were unstable at lower frequencies in MSS cases (**Fig. 4a**,**b**); we observed a correlation between the frequency of MSI events in MSI-H and MSS malignancies of the same

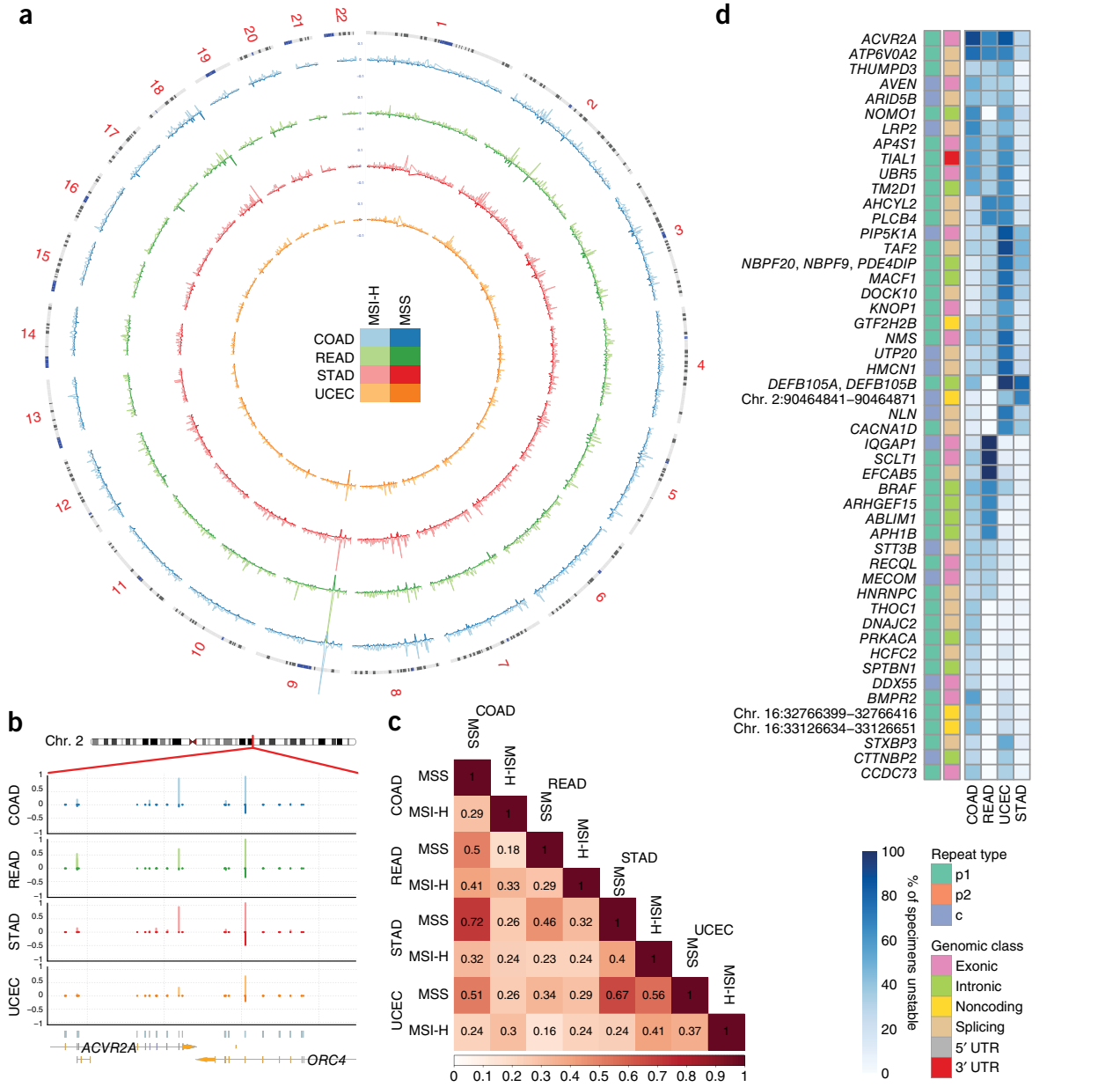


Figure 4 Signatures of MSI in MSI-H tumors. (**a**) Circos plot representing differences in the proportion of MSS and MSI-H tumors with instability at microsatellite loci across the genome. External ring denotes chromosomal position; internal rings indicate the average proportion of cancers with microsatellite locus instability for markers integrated across 1-Mb windows. For each cancer type, the proportion of tumors with instability within each window is indicated by the height of the trace, with MSI-H and MSS tumors for each cancer type plotted separately on different sides of the axis (positive and negative, respectively). The large peak on chromosome 9 reflects a single microsatellite in an intergenic region between *LINGO1* and *LOC401497* that was frequently unstable in both MSI-H and MSS cancers. (**b**) Representative region of chromosome 2 demonstrating differences in MSI profiles between MSI-H and MSS tumors and differences among MSI-H cancer types. The proportion of tumors with locus instability at each locus is indicated as in **a**. (**c**) Cosine similarity matrix comparing the sets of microsatellites unstable in at least half of tumors in each cancer subtype (stratified by MSI status and cancer type). (**d**) Heatmap indicating the proportion of specimens within MSI-H cancer types affected (columns) at the top 50 most differentially unstable microsatellite loci (rows). All loci were significant at FDR < 10^{-5}. For each microsatellite, repeat type and genomic annotation are indicated. p1, mononucleotide repeat; p2, dinucleotide repeat; c, compound repeat. UCEC, uterine corpus endometrial carcinoma (n = 437); COAD, colon adenocarcinoma (n = 294); STAD, stomach adenocarcinoma (n = 278); READ, rectal adenocarcinoma (n = 96).

© 2016 Nature America, Inc. All rights reserved.

Table 1 Ten most significant loci associated with MSI-H cancers

Locus coordinates	Proportion unstable (MSI-H)	Proportion unstable (MSS)	*P* value	*Q* value	OR	Genomic class	Gene(s)	Repeat sequence
Chr. 8:7679723–7679741	190/263 (72%)	173/5626 (3%)	9.19×10^{-191}	1.88×10^{-185}	81.76	Intronic	*DEFB105A*, *DEFB105B*	(A)9
Chr. 2:148683681–148683698	134/253 (52%)	30/5504 (<1%)	1.97×10^{-168}	2.02×10^{-163}	203.24	Coding	*ACVR2A*[a]	(A)8
Chr. 8:7346862–7346880	188/263 (71%)	274/5625 (4%)	3.55×10^{-161}	2.42×10^{-156}	48.849	Intronic	*DEFB105A*, *DEFB105B*	(T)9
Chr. 17:56435156–56435172	112/243 (46%)	10/5557 (<1%)	6.86×10^{-154}	3.51×10^{-149}	471.32	Coding	*RNF43*[a]	(C)7
Chr. 3:51417599–51417615	109/233 (46%)	24/4824 (<1%)	3.55×10^{-133}	1.46×10^{-128}	174.42	Coding	*DOCK3*[a]	(C)7
Chr. 7:74608736–74608758	230/257 (89%)	873/4882 (17%)	3.17×10^{-129}	1.08×10^{-124}	39.1	ncRNA Intronic	*GTF2IP1*, *LOC100093631*	(T)13
Chr. 11:120350632–120350654	104/264 (39%)	29/5579 (<1%)	1.31×10^{-121}	3.82×10^{-117}	124.15	Intronic	*ARHGEF12*[a]	(T)8(C)5
Chr. 16:14983087–14983105	100/264 (37%)	25/5643 (<1%)	5.99×10^{-119}	1.53×10^{-114}	136.12	Intronic	*NOMO1*[a]	(A)9
Chr. 1:151196698–151196722	127/264 (48%)	110/5643 (1%)	6.84×10^{-119}	1.56×10^{-114}	46.56	Coding	*PIP5K1A*[a]	(T)9(C)6
Chr. 1:200594037–200594054	98/250 (39%)	23/5249 (<1%)	2.89×10^{-117}	5.91×10^{-113}	145.57	Intergenic	*KIF14*[a] (dist. = 4,175 bp), *DDX59* (dist. = 19,111 bp)	(T)8

Dist., distance from microsatellite to indicated gene.
[a]Gene is implicated in oncogenesis (**Supplementary Table 12**).

cancer type and also across types (ρ = 0.28–0.53), indicating related instability patterns within and across malignancies. A representative example is provided by two loci in neighboring genes *ACVR2A* (chr. 2:148683681–148683698) and *ORC4* (chr. 2:148701095–148701119) (**Fig. 4b**). *ACVR2A* contains a coding mononucleotide microsatellite that is unstable in 28–90% of MSI-H samples (**Supplementary Table 10**), depending on the cancer type, but in only 0–6% of MSS cancers. *ORC4* harbors a mononucleotide repeat in a splicing region that is also unstable in 67–100% of MSI-H tumors but in only 19–44% of MSS samples.

To examine differences between MSS and MSI-H categories, we focused on microsatellites that were unstable in at least 25% of the samples within each cancer subtype and typable in all specimens. We computed cosine similarities between sets of all frequently unstable sites between each group (**Fig. 4c**). Colon, rectal, gastric, and endometrial MSI-H cancers intersected at a large fraction of their frequently unstable microsatellites, with tissue-matched MSS cancers sharing a smaller subset of those loci. MSS tumors from different tumor types showed substantially less overlap. Taken together, these findings indicate that MSI patterns in tissue-matched MSI-H and MSS cancers are related and follow consistent patterns, but MSI-H cancers share overall similarities in their most frequently unstable sites.

Differences among MSI-H cancers

To compare MSI among different MSI-H cancer types, we examined only cancer types with the highest MSI-H prevalence to lend sufficient power for statistically meaningful comparisons. As was observed for the entire collection of specimens (**Fig. 3**), separate MSI-H cancer types showed individualized signatures of instability at a subset of microsatellite loci (**Fig. 4d**). In total, 2,685 of the 3,296 microsatellites unstable in at least 5% of MSI-H cancers were differentially unstable in at least one cancer type at an FDR < 0.05 (**Supplementary Table 10**). These differentially unstable microsatellites included several in *NIPBL*, *TCF4*, and *PTEN*, among other genes reported as mutational targets of MSI[25,26]. An example is again provided by the microsatellites in *ACVR2A* and *ORC4* (**Fig. 4b**): the former was unstable in 90% of colon, 67% of rectal, and 87% of stomach MSI-H tumors, but only 28% of endometrial MSI-H tumors, and the latter was unstable in 97% of colon, 67% of endometrial, and 100% of rectal and stomach MSI-H tumors investigated.

To explore the functional consequences of different instability signatures among MSI-H cancer types, we examined factors that were uniquely unstable in one cancer type (**Supplementary Fig. 7**). Uniquely unstable factors in colon and rectal cancers shared overlap in multiple aggregated functional categories, although cancer-type-specific differences were observed among assorted cellular functions. Stomach adenocarcinomas were uniquely and highly enriched for instability in ion-binding genes while demonstrating instability in several categories frequently observed for MSI-H colon and rectal tumors. Endometrial cancers were exclusively enriched for uniquely unstable sites in protein complex binding genes, without overlap in categories identified for other cancer types, although the small number of endometrial-cancer-specific unstable sites limited our power for ascertaining such ontological enrichments.

Properties of unstable microsatellites

We investigated features associated with unstable loci by associating various intrinsic properties, annotations, and metrics with the likelihood of locus instability. After stratifying by repeat composition and MSI status, we found compound microsatellites to be more preferentially unstable than other repeat types, with 11.7% and 5.3% of those loci unstable in more than 20% of MSI-H and MSS samples, respectively (**Supplementary Fig. 8a**). Intrinsic length of the microsatellite tract had bearing on instability frequency, with a maximum occurring around 16 repeat units in length (**Supplementary Fig. 8b**). When loci were stratified by their genomic annotations (**Supplementary Fig. 8c**), microsatellites in coding regions were less likely to be unstable in at least one sample (OR = 0.87, $P = 2.3 \times 10^{-57}$). By contrast, microsatellites in splice sites were more likely to be unstable (OR = 1.37, $P = 2.2 \times 10^{-82}$). We compared primary sequence enrichments of microsatellites unstable in at least one cancer (**Supplementary Fig. 8d**) and observed no significant differences among MSI-H cancer types (**Supplementary Fig. 9a**). However, CA and GA dinucleotide repeats were the most likely to be unstable overall. We also observed variability in the likelihood of instability at CpG sites, which probably reflects their functional importance in gene regulation. We observed significant enrichments for instability at DNase hypersensitivity sites ($P = 0.01$), conserved transcription factor binding sites ($P = 3 \times 10^{-6}$), and evolutionarily conserved genomic regions ($P = 6 \times 10^{-9}$; **Supplementary Fig. 9b**). Last, we tested for a correlation between the average frequency of locus instability within 1-Mb windows across individuals and DNA replication timing[10,27] but found no significant associations (**Supplementary Fig. 9c**).

Unstable microsatellites in cancer-associated genes

To identify elements common to a generalizable MSI-H signature across cancer types, we tested for loci that were significantly more

© 2016 Nature America, Inc. All rights reserved.

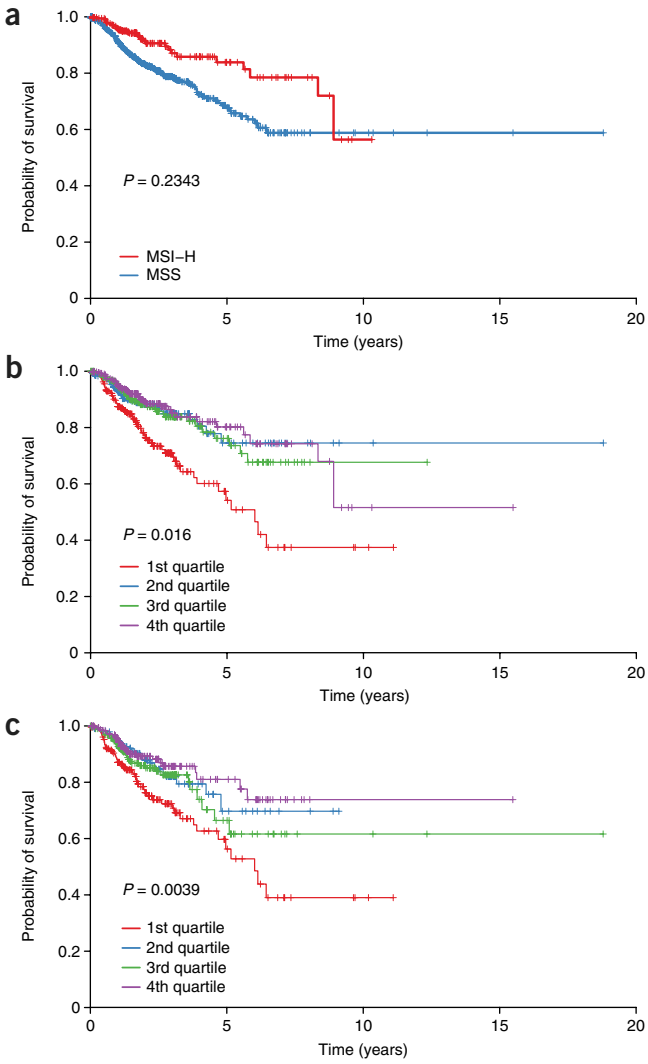


Figure 5 Global MSI load and patient survival. (**a**) Patient survival aggregated for endometrial, stomach, colon, and rectal cancers, stratified by inferred MSI status (MSS $n = 864$, MSI-H $n = 241$). (**b**) Patient survival for the same tumors in **a** as a function of the proportion of unstable microsatellites detected, grouped by quartile. (**c**) Patient survival for MSS cancers from **a**, grouped by quartile. *P* values in **b** and **c** represent the significance of the continuous variable of the proportion of unstable microsatellites per sample as determined from likelihood ratio tests. Significance in all panels was assessed after correcting for age, sex, radiation therapy status, and cancer type.

likely to be unstable in all MSI-H tumors ($n = 264$) than in all MSS cancers ($n = 5{,}666$). Of the 204,797 microsatellites with sufficient coverage to be called in at least half of MSI-H and MSS samples across cancer types, 17,564 sites within 6,882 unique genes were significant at an FDR < 0.05 (**Supplementary Fig. 8e** and **Supplementary Table 11**), indicating that a subset of markers are reliably unstable across cancer types and may represent common genomic lesions in MSI-H malignancies.

We noted that many recurrently unstable loci in MSI-H tumors (**Table 1**) involved cancer-associated genes, including coding regions in tumor suppressor genes *ACVR2A* and *RNF43*, which are frequent and validated targets of mutation in MSI-H cancers[28,29]. We explored a possible correlation of instability events with occurrence in genes participating in oncogenic pathways[9]. Using permutation testing, we tested whether recurrently unstable loci (**Supplementary Table 10**) were more likely to occur in genes registered in the COSMIC cancer gene census[30] and observed that microsatellites located in genes with known involvement in oncogenesis were significantly more likely to be unstable (**Supplementary Fig. 8f**; OR = 1.51, $P < 10^{-4}$). Moreover, a review of the literature for the genes harboring or proximal to the top 100 most significantly mutated loci in MSI-H cancers showed that 58 are in or near genes with previously established cancer-related biological functions (**Supplementary Table 12**). Furthermore, 25 of 27 known recurrent mutational targets of colorectal cancer MSI[26] examined in our study contained loci that were significantly unstable in MSI-H relative to MSS samples at an FDR < 0.05 ($P = 5 \times 10^{-9}$).

Patient survival and MSI burden

MSI-H status is associated with modestly improved patient survival in colorectal cancers[31]. We therefore examined whether there was a general correlation between MSI-H classification and survival outcome across cancer types, after correcting for covariates. We observed a weak association between MSI status and survival outcome when considering in aggregate the four cancer types with the highest incidence of MSI-H ($P = 0.23$, hazard ratio (HR) for MSI-H = 0.79; **Fig. 5a**). We next evaluated whether the global burden of unstable microsatellites would correlate with survival when treated as a continuous variable independently of MSI status and observed a stronger, more significant positive correlation with survival ($P = 0.02$, HR per increase of 100 unstable sites = 0.984; **Fig. 5b**). Given that MSI-H samples showed, on average, approximately 2,100 more unstable sites than MSS samples, this would equate to a HR of 0.72 for MSI-H. Furthermore, the association between the number of unstable sites and patient survival was more pronounced in MSS samples alone ($P = 0.004$, HR per increase of 100 unstable sites = 0.959; **Fig. 5c**). This observation led us to question whether the metric would also be prognostic of patient outcome in cancer types for which MSI is not typically evaluated. Although no significant effect was observed when cancer types were examined in aggregate, for individual cancer types we observed positive trends between prognosis and instability burden in uterine, endometrial, rectal, colon, stomach, and thyroid cancer and lower-grade glioma (**Supplementary Fig. 10**). Limited sample sizes for each cancer type restrict power for establishing the significance of these trends.

Last, we tested whether MSI was high in cancers that had progressed by quantifying instability events in primary and metastatic tumors within cancer types. We examined cancers for which multiple patient samples from metastatic disease were available, including seven patient-matched metastatic and primary breast tumors, seven patient-matched metastatic and primary thyroid tumors, and six primary and 174 metastatic melanoma cases from unrelated patients. All were MSS. The fractions of unstable loci were not significantly different between metastatic and primary tumors (median percentage unstable for each group = 0.37%, $P = 0.13$, nested ANOVA; **Supplementary Fig. 11**), which suggests that MSI is not associated with likelihood of metastasis, although additional samples will be necessary to substantiate this observation.

DISCUSSION

To explore the landscape of MSI in different cancers, we developed MOSAIC for ascertaining MSI status from tumor–normal tissue pairs examined with exome-sequencing data. Our approach leverages the observation that MSS tumors have a lower baseline level of instability

© 2016 Nature America, Inc. All rights reserved.

events than MSI-H tumors, which enables MSI classifications to be distinguished on the basis of global MSI calls. MOSAIC corrects for class imbalance in its cross-validation training procedure (an approximately 3:1 MSS-to-MSI-H ratio), allowing predictions in new cancer types to be made without prior assumption about the expected prevalence of MSI-H tumors. Although we noted a few discrepancies between our classifier and conventional MSI typing, genomic data suggest that these represent false positive and false negative outcomes from clinical typing[32,33] and that discordant results are more consistent with MOSAIC classifications.

Most cancer types examined (14 of 18) included one or more MSI-H representatives, suggesting that MSI may be a generalized cancer phenotype. The identification of infrequently occurring MSI-H tumors from cancer types conventionally associated with MSI confirms published reports[6,34–40]. Notably, most cancer types, even those for which there were few or no examples with the MSI-H phenotype in our cohort, showed a high frequency of MSI at restricted subsets of loci. This observation raises the possibility that findings[41] of MSI in some cancer types may reflect artifacts from typing local mutational hot spots by conventional methods rather than a global instability phenotype.

Microsatellite mutations occurring within the coding regions, introns, or untranslated regions of genes may positively or negatively influence gene expression or protein function by affecting changes in transcription or gene splicing[9,15,42–44]. We observed a depletion of unstable microsatellites in exons, transcription factor binding sites, and evolutionarily conserved genomic regions, consistent with purifying selection against mutations with biologically functional consequences[10]. Nevertheless, regulatory alterations for some targets may confer selective growth advantages to cancer cells, and unstable microsatellite loci have been speculated to fall within genes implicated in oncogenesis and to participate in the evolution of MSI-H cancers[9,13,14,16,45–47]. For unstable microsatellites observed in genic regions, our data support the idea that they preferentially accumulate in genes involved in carcinogenesis or tumor survival and therefore probably serve as drivers of cancer evolution. Differences in patterns of MSI among cancer types may consequently reflect different positive and negative selective pressures experienced during carcinogenesis. We observed that frequently unstable microsatellites in MSI-H malignancies are preferentially located in known cancer-associated genes, supporting this view and suggesting that there may be an underappreciated contribution of MSI in generating cancer-driving mutations. Moreover, roughly half of unstable microsatellites fall within genes not previously reported to be involved in cancer, including several intergenic loci, raising the possibility that these microsatellites also function as cancer drivers. Although functionally evaluating newly implicated factors is outside the scope of this work, many of the differences between MSS and MSI-H tumors are pronounced, and these data illustrate the utility of microsatellite analysis of exome-sequencing data as a primary approach for identifying cancer-relevant genes. Identification of features that are recurrently affected by MSI is complementary to methods that highlight genes on the basis of their recurrent somatic coding sequence mutations[48].

Although differences in selection during carcinogenesis may account for much of the variability in instability rates observed among microsatellite markers, we also observed significant correlations with more generalized properties of the loci themselves. We observed a weak but significant correlation between elevated MSI rates and loci occupying DNase-hypersensitivity sites, supporting earlier work[10] and indicating that instability events are enriched within euchromatic regions. Other factors, including repeat composition and locus length, affected instability[44]. It is likely that local nucleotide sequence or secondary structure surrounding repeats also define the inherent instability of a locus[44,49].

Consistent with other genomic studies[10,21], we found no evidence that tumors classified as MSI-L are a distinct disease group. This conclusion supports the view that MSI-L is a technical artifact reflecting a low background frequency of MSI in tumors with intact MMR systems[1,24]. Nevertheless, specimens in our study spanned a continuum of observed instability, and, at their extremes, tumors classified as MSI-H and MSS showed some overlap in their overall burden of unstable microsatellites. In general, we observed that the number of unstable microsatellite loci in a tumor exome correlated with patient survival when considered as a continuous metric better than conventional MSI-H or MSS classification alone. This result may reflect a link between MSI events and the production of cancer neoantigens that can be recognized as 'non-self' by the immune system[7,50]. Although the effect sizes we observed were smaller because of our limited cohort sizes, they are consistent with values reported in larger cohorts[31]. These findings suggest that, when sufficient numbers of loci are considered, the MSI phenotype may be a more continuous phenotype than previously appreciated—indeed, the global burden of MSI within MSS samples alone was prognostic of patient outcome. Because this continuous distribution of global instability is more indicative of patient survival independently of conventional MSI classification, it may prove more informative in the clinical management and treatment of cancer[7].

The existence of cancer-specific MSI landscapes and the potential predictive power of MSI as a continuous metric have implications for the molecular diagnosis of MSI in clinical practice: because current assays are optimized for the detection of MSI in colon and rectal cancers[17], they may not detect instability events effectively, or at all, in other cancer types. The behavior of any particular microsatellite locus can vary greatly across cancers, and loci that are inherently stable in one cancer type may be frequently mutated in another. Because MOSAIC for genome-scale MSI classification is more comprehensive and less prone to cancer-type-specific biases, it may serve as a better clinical strategy for pan-cancer MSI determination and ascertainment of instability burden.

Microsatellites are preferentially located in noncoding regions of the genome, and we anticipate that the future availability of more cancer whole-genome sequences will provide an improved understanding of the overall genomic landscape of MSI in different malignancies. As suggested by our study, such data may implicate novel, noncoding oncogenic motifs that affect gene regulation and will yield further insights into potentially important genomic sites involved in carcinogenesis.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank A. McKenna and other members of S.J.S. and J.S.'s laboratories for helpful advice and assistance. This work was supported in part by the Damon Runyon Cancer Research Foundation (DRG-2224-15 to R.J.H.), a Congressionally Directed Medical Research Program (PC131820 to C.C.P.), and a Young Investigator Award from the Prostate Cancer Foundation (to C.C.P.).

© 2016 Nature America, Inc. All rights reserved.

AUTHOR CONTRIBUTIONS

R.J.H. and S.J.S. conceived the work and designed and performed the analyses; R.J.H., S.J.S., C.C.P., and J.S. interpreted results; and R.J.H. and S.J.S. wrote the paper with input from C.C.P. and J.S.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. de la Chapelle, A. & Hampel, H. Clinical relevance of microsatellite instability in colorectal cancer. *J. Clin. Oncol.* **28**, 3380–3387 (2010).
2. Murphy, K.M. *et al.* Comparison of the microsatellite instability analysis system and the Bethesda panel for the determination of microsatellite instability in colorectal cancers. *J. Mol. Diagn.* **8**, 305–311 (2006).
3. Vilar, E. & Gruber, S.B. Microsatellite instability in colorectal cancer—the stable evidence. *Nat. Rev. Clin. Oncol.* **7**, 153–162 (2010).
4. Oki, E., Oda, S., Maehara, Y. & Sugimachi, K. Mutated gene-specific phenotypes of dinucleotide repeat instability in human colorectal carcinoma cell lines deficient in DNA mismatch repair. *Oncogene* **18**, 2143–2147 (1999).
5. Boland, C.R. *et al.* A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res.* **58**, 5248–5257 (1998).
6. Pritchard, C.C. *et al.* Complex MSH2 and MSH6 mutations in hypermutated microsatellite unstable advanced prostate cancer. *Nat. Commun.* **5**, 4988 (2014).
7. Le, D.T. *et al.* PD-1 blockade in tumors with mismatch-repair deficiency. *N. Engl. J. Med.* **372**, 2509–2520 (2015).
8. Timmermann, B. *et al.* Somatic mutation profiles of MSI and MSS colorectal cancer identified by whole-exome next-generation sequencing and bioinformatics analysis. *PLoS One* **5**, e15661 (2010).
9. Woerner, S.M. *et al.* SelTarbase, a database of human mononucleotide-microsatellite mutations and their potential impact to tumorigenesis and immunology. *Nucleic Acids Res.* **38**, D682–D689 (2010).
10. Kim, T.M., Laird, P.W. & Park, P.J. The landscape of microsatellite instability in colorectal and endometrial cancer genomes. *Cell* **155**, 858–868 (2013).
11. Onda, M. *et al.* Microsatellite instability in thyroid cancer: hot spots, clinicopathological implications, and prognostic significance. *Clin. Cancer Res.* **7**, 3444–3449 (2001).
12. Forgacs, E. *et al.* Searching for microsatellite mutations in coding regions in lung, breast, ovarian and colorectal cancers. *Oncogene* **20**, 1005–1009 (2001).
13. Duval, A. *et al.* Target gene mutation profile differs between gastrointestinal and endometrial tumors with mismatch repair deficiency. *Cancer Res.* **62**, 1609–1612 (2002).
14. Mori, Y. *et al.* Instabilotyping reveals unique mutational spectra in microsatellite-unstable gastric cancers. *Cancer Res.* **62**, 3641–3645 (2002).
15. Sonay, T.B., Koletou, M. & Wagner, A. A survey of tandem repeat instabilities and associated gene expression changes in 35 colorectal cancers. *BMC Genomics* **16**, 702 (2015).
16. Yoon, K. *et al.* Comprehensive genome- and transcriptome-wide analyses of mutations associated with microsatellite instability in Korean gastric cancers. *Genome Res.* **23**, 1109–1117 (2013).
17. Bacher, J.W. *et al.* Development of a fluorescent multiplex assay for detection of MSI-high tumors. *Dis. Markers* **20**, 237–250 (2004).
18. Lu, Y., Soong, T.D. & Elemento, O. A novel approach for characterizing microsatellite instability in cancer cells. *PLoS One* **8**, e63056 (2013).
19. McIver, L.J., Fonville, N.C., Karunasena, E. & Garner, H.R. Microsatellite genotyping reveals a signature in breast cancer exomes. *Breast Cancer Res. Treat.* **145**, 791–798 (2014).
20. Niu, B. *et al.* MSIsensor: microsatellite instability detection using paired tumor-normal sequence data. *Bioinformatics* **30**, 1015–1016 (2014).
21. Salipante, S.J., Scroggins, S.M., Hampel, H.L., Turner, E.H. & Pritchard, C.C. Microsatellite instability detection by next generation sequencing. *Clin. Chem.* **60**, 1192–1199 (2014).
22. Huang, M.N. *et al.* MSIseq: software for assessing microsatellite instability from catalogs of somatic mutations. *Sci. Rep.* **5**, 13321 (2015).
23. Pawlik, T.M., Raut, C.P. & Rodriguez-Bigas, M.A. Colorectal carcinogenesis: MSI-H versus MSI-L. *Dis. Markers* **20**, 199–206 (2004).
24. Laiho, P. *et al.* Low-level microsatellite instability in most colorectal carcinomas. *Cancer Res.* **62**, 1166–1170 (2002).
25. Kim, M.S., An, C.H., Chung, Y.J., Yoo, N.J. & Lee, S.H. NIPBL, a cohesion loading factor, is somatically mutated in gastric and colorectal cancers with high microsatellite instability. *Dig. Dis. Sci.* **58**, 3376–3378 (2013).
26. Boland, C.R. & Goel, A. Microsatellite instability in colorectal cancer. *Gastroenterology* **138**, 2073–2087 (2010).
27. Supek, F. & Lehner, B. Differential DNA mismatch repair underlies mutation rate variation across the human genome. *Nature* **521**, 81–84 (2015).
28. Jung, B. *et al.* Loss of activin receptor type 2 protein expression in microsatellite unstable colon cancers. *Gastroenterology* **126**, 654–659 (2004).
29. Giannakis, M. *et al.* RNF43 is frequently mutated in colorectal and endometrial cancers. *Nat. Genet.* **46**, 1264–1266 (2014).
30. Futreal, P.A. *et al.* A census of human cancer genes. *Nat. Rev. Cancer* **4**, 177–183 (2004).
31. Samowitz, W.S. *et al.* Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. *Cancer Epidemiol. Biomark. Prev.* **10**, 917–923 (2001).
32. Hampel, H. *et al.* Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients. *Cancer Res.* **66**, 7810–7817 (2006).
33. Goel, A., Nagasaka, T., Hamelin, R. & Boland, C.R. An optimized pentaplex PCR for detecting DNA mismatch repair–deficient colorectal cancers. *PLoS One* **5**, e9393 (2010).
34. Altavilla, G., Fassan, M., Busatto, G., Orsolan, M. & Giacomelli, L. Microsatellite instability and hMLH1 and hMSH2 expression in renal tumors. *Oncol. Rep.* **24**, 927–932 (2010).
35. Martinez, R. *et al.* Low-level microsatellite instability phenotype in sporadic glioblastoma multiforme. *J. Cancer Res. Clin. Oncol.* **131**, 87–93 (2005).
36. Jensen, K.C. *et al.* Microsatellite instability and mismatch repair protein defects in ovarian epithelial neoplasms in patients 50 years of age and younger. *Am. J. Surg. Pathol.* **32**, 1029–1037 (2008).
37. Kazachkov, Y. *et al.* Microsatellite instability in human hepatocellular carcinoma: relationship to p53 abnormalities. *Liver* **18**, 156–161 (1998).
38. Dacic, S., Lomago, D., Hunt, J.L., Sepulveda, A. & Yousem, S.A. Microsatellite instability is uncommon in lymphoepithelioma-like carcinoma of the lung. *Am. J. Clin. Pathol.* **127**, 282–286 (2007).
39. Field, J.K. *et al.* Microsatellite instability in squamous cell carcinoma of the head and neck. *Br. J. Cancer* **71**, 1065–1069 (1995).
40. Eckert, A. *et al.* Microsatellite instability in pediatric and adult high-grade gliomas. *Brain Pathol.* **17**, 146–150 (2007).
41. Amira, N. *et al.* Microsatellite instability in urothelial carcinoma of the upper urinary tract. *J. Urol.* **170**, 1151–1154 (2003).
42. Dorard, C. *et al.* Expression of a mutant HSP110 sensitizes colorectal cancer cells to chemotherapy and improves disease prognosis. *Nat. Med.* **17**, 1283–1289 (2011).
43. Shin, N. *et al.* Identification of frequently mutated genes with relevance to nonsense-mediated mRNA decay in the high microsatellite instability cancers. *Int. J. Cancer* **128**, 2872–2880 (2011).
44. Shah, S.N., Hile, S.E. & Eckert, K.A. Defective mismatch repair, microsatellite mutation bias, and variability in clinical cancer phenotypes. *Cancer Res.* **70**, 431–435 (2010).
45. Woerner, S.M. *et al.* Pathogenesis of DNA repair-deficient cancers: a statistical meta-analysis of putative Real Common Target genes. *Oncogene* **22**, 2226–2235 (2003).
46. Duval, A. *et al.* Evolution of instability at coding and non-coding repeat sequences in human MSI-H colorectal cancers. *Hum. Mol. Genet.* **10**, 513–518 (2001).
47. Imai, K. & Yamamoto, H. Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. *Carcinogenesis* **29**, 673–680 (2008).
48. Lawrence, M.S. *et al.* Discovery and saturation analysis of cancer genes across 21 tumor types. *Nature* **505**, 495–501 (2014).
49. Eckert, K.A. & Hile, S.E. Every microsatellite is different: Intrinsic DNA features dictate mutagenesis of common microsatellites present in the human genome. *Mol. Carcinog.* **48**, 379–388 (2009).
50. Mlecnik, B. *et al.* Integrative analyses of colorectal cancer show immunoscore is a stronger predictor of patient survival than microsatellite instability. *Immunity* **44**, 698–711 (2016).

© 2016 Nature America, Inc. All rights reserved.

ONLINE METHODS

Exome microsatellite data. Exome data for all specimens (tumors and patient-matched normal blood) were obtained from the TCGA Research Network (http://cancergenome.nih.gov/; **Supplementary Table 5**) as alignments against hg19. Researchers were not blinded to the MSI status of specimens where those data were available. We identified all autosomal microsatellite tracts with repeating subunits of 1–5 bp in length and comprising 5 repeats or more in the human reference genome (GRCh37/hg19) using MISA (http://pgrc.ipk-gatersleben.de/misa/misa.html) and padded their start and stop coordinates by 5 bp. 10 bp or fewer were permitted between repeats for adjacent microsatellites to be combined into single loci, termed either 'complex' (c*) if comprised of microsatellites with different repeat subunit lengths or 'compound' (c) if comprised of disparate repeats with the same repeat length. Microsatellites directly tiled by the NimbleGen SeqCap_EZ_Exome_v3 capture design (which was used by TCGA) and those within 50 bp of a capture bait were retained. Repeat features were annotated using ANNOVAR[51] (24 February 2014 release).

Calling unstable microsatellite loci. Primary analysis of microsatellite loci was performed in each specimen to determine stability using mSINGS as previously described[21]. Briefly, we evaluated the number of sequence reads of different lengths present within each of the identified microsatellite markers, then expressed the relative abundance of individual lengths for a microsatellite as the fraction of reads supporting that length normalized to the number of reads counted for the most frequently occurring length at that locus. Microsatellite tract lengths at <5% relative abundance were discarded. Although identified length polymorphisms may include some reproducible artifacts resulting from slippage during PCR amplification, their total number is proportional to the actual number of microsatellite alleles present at a locus[21], and in comparative analysis of genetically related tumor–normal pairs such artifacts are well controlled. Instability at each locus was subsequently defined in two ways: (i) the high-sensitivity approach, in which identification was performed by comparing the absolute number of lengths identified between tumor and paired normal specimens, and the locus was considered unstable if one or more additional lengths for a microsatellite were detected from the tumor; and (ii) the high-specificity approach, in which Kolmogorov–Smirnov scores were calculated when comparing the normalized distribution of lengths for tumor and paired normal specimens, considering any difference less than $P = 0.05$ to signify locus instability[10]. We determined the latter method to be overly conservative (a median of only 5 unstable sites were called per MSI-H cancer), and therefore did not implement it in practice. Accordingly, the burden of unstable sites identified in our study was considerably higher than approximated in other work[10], probably because of the greater sensitivity-to-specificity tradeoff of our approach.

Constructing MOSAIC from sequencing-based locus instability calls. We examined data from colon, rectal, stomach, and endometrial cancer exomes (**Supplementary Tables 4** and **5**) for which clinical MSI status was available from standard diagnostic methods[17]. We observed that the average size of instability events (i.e., the length of alternate microsatellite alleles) was greater in MSI-H than MSS tumors (**Fig. 1d**,**e**; $P = 9 \times 10^{-80}$, two-sided Wilcoxon rank-sum test). Clinical MSI–PCR results (MSI-H, MSI-L, and MSS) were obtained from TCGA. The average gain in unique alleles in tumor relative to matched normal tissue across all interrogated microsatellites (peak_avg), variation in allele gain (peak_var), total number of unstable sites defined by the high-sensitivity method (num_unstable), and proportion of callable unstable sites (prop_unstable) were calculated for each sample. Furthermore, we tested for the power of each microsatellite locus to differentiate between MSI-H and MSS tumors using Fisher's exact tests and identified a locus within *DEFB105A/B*, chr. 8:7679723–7679741, as the most significantly unstable microsatellite in MSI-H relative to MSS tumors (defbsite). These features, along with the top 100 most significantly unstable microsatellites in MSI-H relative to MSS tumors, were then used to predict clinical MSI-H or MSS diagnosis by recursive partitioning classification trees or random forests implemented using the rpart v4.1-10, randomForest v4.6-12, and caret v6.0.62 packages in R v3.2.1. Leave-one-sample-out cross-validation was used to learn the optimal features and parameters for predicting MSI status, interrogating a grid search space of 0, 0.001, 0.01, 0.1, 0.45, and 0.95 complexity parameters (cp) with the minimum number of observations in any terminal node (minbucket) set to 6 and the maximum depth of any node of the final tree set to 3 for recursive partitioning, and 2 and 3 randomly sampled variables as candidates at each split (mtry) with 1,000 trees for random forests. Weights were included to correct for class imbalances in the training data (MSI-H $n = 171$, MSS $n = 446$), and the optimal parameters selected were cp = 0.001 and mtry = 2. Notably, peak_avg and debsite were selected by recursive feature selection using decision trees as the most significant two features for inclusion in the final model; incorporating more than two covariates did not significantly improve the classifier (**Supplementary Fig. 3b**). The final models achieved 96.6% (rpart) and 96.4% (randomForest) accuracy. The more accurate and parsimonious rpart model was used to predict MSI status across all remaining cancer samples.

Identifying uniquely unstable microsatellites in MSI-H cancers. For each of the 204,797 microsatellite loci called in at least half of MSI-H and MSS cancers ($n > 132$ and $n > 2{,}833$, respectively), we performed two-sided Fisher exact tests comparing the ratios of individuals for which the site was unstable in MSI-H samples to the ratio of individuals for which the site was unstable in MSS samples. FDR values were estimated using Storey's q-value method, with a q-value < 0.05 considered significant.

Determining cancer-specific microsatellite sites. Multiple proportions tests were implemented in R using the prop.test function to identify sites differentially unstable in at least one cancer type relative to the average frequency of instability observed across all other groups from the 92,385 sites called in at least half of the samples for each cancer. To determine MSI-H cancer-specific microsatellites, multiple proportions tests were performed for each site for colon, rectal, endometrial, and stomach MSI-H cancers. FDR values were estimated as described above. To compare across cancers and MSI diagnostic types, we computed cosine similarity scores. Because the number of frequently unstable sets in MSI-H cancers was an order of magnitude larger than that observed for MSS cancers, cosine similarity was less sensitive to these set inequalities than the overlap coefficient or Jaccard index, which artificially inflate or deflate the observed overlap, respectively.

Gene Ontology enrichment analyses. Gene enrichment was performed using the R package clusterProfiler version 2.2.5 (ref. 52). clusterProfiler implements a hypergeometric model to test for gene set overrepresentation relative to a background gene set. Each cluster (1–4) from the global instability results was compared with the background of all other microsatellites sequenced at sufficient depth in our study, with a Benjamini–Hochberg FDR threshold of 0.20 defined as significant enrichment. Enrichment in KEGG pathways was analyzed with the enrichKEGG function and the same parameters. Enrichment between MSI-H specific clusters was analyzed using the compareCluster function with fun = enrichGO, pvalueCutoff = 0.05, OrgDb = org.Hs.eg.db. Significantly enriched GO terms were simplified using GOSemSim to calculate the similarity of GO terms and remove highly similar terms (cutoff = 0.7) by retaining the most significant representative term. GO analyses were corrected for gene size in that enrichment analyses were performed at the microsatellite level, such that larger genes required greater numbers of unstable sites for significant enrichment relative to the background distributions of microsatellites in genes covered in our study.

Enrichment of unstable microsatellites in cancer-associated genes. After excluding microsatellites with intergenic and intronic annotations, we extracted annotations for the 252,127 microsatellites that had valid calls in MSS samples, resulting in a panel of 18,104 unique genes. We compared this full gene panel against the 17,564 loci that were unstable with significantly greater frequency in MSI-H cancers at an FDR < 0.05, comprising a set of 6,821 unique genes. We compared these data sets to the COSMIC cancer gene census (accessed 15 June 2015), which contained 573 unique cancer-associated genes. To test for enrichment against the COSMIC database, 1,000 permutations were performed, sampling 6,821 genes from all possible unique genes in the full gene panel.

Correlation of instability and DNA replication timing. We first filtered our data to 77,215 sites that were called in more than half of the samples within each

© 2016 Nature America, Inc. All rights reserved.

doi:10.1038/nm.4191

of the 32 (cancer type by MSI status) groups and called completely across all groups. We downloaded wavelet-smoothed Repli-Seq signals from 11 ENCODE cell lines from the UCSC Genome Browser (GEO GSE34399). We then averaged the proportions of MSI and Repli-Seq signals across 1-Mb windows throughout the genome and calculated the median Repli-Seq signal across all 11 cell lines as representative of 'general' replication timing throughout the genome, with values ranging 0–100 (higher numbers indicating earlier replication). Spearman correlation coefficients were calculated between binned, averaged instability proportions between MSI classifications and across cancer types compared with median and cell-line-specific binned Repli-Seq signals.

Survival analyses. We assessed the association of MSI with overall survival using the coxph function from the R survival package version 2.38, with significance assessed by Wald tests. Age, sex, cancer type, radiation therapy, and pathologic stage (I, II, III, IV) were included as covariates in multivariate analyses. The proportional hazards assumption for covariates in these Cox regression models was tested using the cox.zph function and violating covariates were stratified when necessary.

Statistical analyses. All statistical tests used in this study were nonparametric and therefore made no assumptions about distributions or equal variance between groups. Two-sided Fisher exact tests were used to identify differentially unstable microsatellites in MSI-H cancers and enriched or depleted genomic annotations for unstable sites. To determine unstable microsatellites unique to specific MSI-H cancers, multiple proportions tests were performed for each site across colon, rectal, endometrial, and stomach MSI-H cancers. FDR values for both analyses were estimated using Storey's q-value method, with a q-value < 0.05 considered significant. To compare instability events across cancers and MSI diagnostic types, we computed cosine similarity scores. Hypergeometric tests were implemented to test for the enrichment of genes harboring frequently unstable sites in GO terms and KEGG pathways. Permutation tests were performed to test for enrichment in MSI-affected genes against the COSMIC database. Spearman correlation coefficients were calculated to evaluate correlations between instability and DNA replication timing. Lastly, survival curves were represented with Kaplan-Meier curves, with the significance of covariate effects estimated by fitting Cox proportional-hazards regression models.

Data access. Primary sequencing data are available from TCGA Research Network (http://cancergenome.nih.gov/). Primary MSI calls from this study are available from (http://krishna.gs.washington.edu/content/members/hauser/mosaic/).

Code availability. Code for primary analysis of microsatellite loci through mSINGS (git commit e32b776) is available at https://bitbucket.org/uwlabmed/msings. Code for secondary analyses and MOSAIC are available at https://github.com/ronaldhause/mosaic.

51. Wang, K., Li, M. & Hakonarson, H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res.* **38**, e164 (2010).
52. Yu, G., Wang, L.-G., Han, Y. & He, Q.-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* **16**, 284–287 (2012).

© 2016 Nature America, Inc. All rights reserved.

www.impactjournals.com/oncotarget/ Oncotarget, 2016, Vol. 7, (No. 50), pp: 82504-82510

Research Paper

Mismatch repair deficiency may be common in ductal adenocarcinoma of the prostate

Michael T. Schweizer[1,2,*], Heather H. Cheng[1,2,*], Maria S. Tretiakova[3], Funda Vakar-Lopez[3], Nola Klemfuss[4], Eric Q. Konnick[5], Elahe A. Mostaghel[1,2], Peter S. Nelson[1,4], Evan Y. Yu[1,2], Bruce Montgomery[1,2], Lawrence D. True[3], Colin C. Pritchard[5]

[1]Department of Medicine, Division of Oncology, University of Washington, Seattle, WA, USA

[2]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

[3]Department of Pathology, University of Washington, Seattle, WA, USA

[4]Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

[5]Department of Laboratory Medicine, University of Washington, Seattle, WA, USA

[*]These authors contributed equally to this work

Correspondence to: Michael T. Schweizer, **email:** schweize@uw.edu
Lawrence D. True, **email:** ltrue@uw.edu
Colin C. Pritchard, **email:** cpritch@uw.edu

Keywords: *prostate cancer, ductal adenocarcinoma, hypermutation, mismatch repair, microsatellite instability*

Received: *August 19, 2016* ***Accepted:*** *October 12, 2016* ***Published:*** *October 15, 2016*

ABSTRACT

Precision oncology entails making treatment decisions based on a tumor's molecular characteristics. For prostate cancer, identifying clinically relevant molecular subgroups is challenging, as molecular profiling is not routine outside of academic centers. Since histologic variants of other cancers correlates with specific genomic alterations, we sought to determine if ductal adenocarcinoma of the prostate (dPC) – a rare and aggressive histopathologic variant – was associated with any recurrent actionable mutations. Tumors from 10 consecutive patients with known dPC were sequenced on a targeted next-generation DNA sequencing panel. The median age at diagnosis was 59 years (range, 40–73). Four (40%) patients had metastases upon presentation. Archival tissue from formalin-fixed paraffin-embedded prostate tissue samples from nine patients and a biopsy of a metastasis from one patient with castration-resistant prostate cancer were available for analysis. Nine of 10 samples had sufficient material for tumor sequencing. Four (40%) patients' tumors had a mismatch repair (MMR) gene alteration (*N* = 2, *MSH2*; *N* = 1, *MSH6*; and *N* = 1, *MLH1*), of which 3 (75%) had evidence of hypermutation. Sections of the primary carcinomas of three additional patients with known MMR gene alterations/hypermutation were histologically evaluated; two of these tumors had dPC. MMR mutations associated with hypermutation were common in our cohort of dPC patients. Since hypermutation may predict for response to immune checkpoint blockade, the presence of dPC may be a rapid means to enrich populations for further screening. Given our small sample size, these findings require replication.

INTRODUCTION

Precision oncology entails therapeutic decision-making on the basis of an individual patient's molecular tumor profile. To that end, it is imperative to develop strategies to rapidly identify clinically relevant patient subgroups. While next-generation sequencing technologies have greatly advanced molecular classification, they are not routinely used for prostate cancer and may be costly. Because histological variants can correlate with genomic alterations in other malignancies (e.g. colorectal carcinoma, acute myelogenous leukemia), we hypothesized that distinct prostate cancer histologies may also associate with underlying molecular aberrations – allowing for the rapid identification of patients for further screening [1–5]. In this study, we sought to determine if ductal prostate cancer (dPC) was associated with clinically actionable molecular features.

Ductal prostatic adenocarcinomas (dPC) are an aggressive histopathologic variant of prostate

cancer, characterized by large glands lined by tall, pseudostratified, columnar neoplastic epithelial cells [6]. Approximately 3% of all prostate cancers have at least a component of ductal histology, with only 0.2% having pure ductal histology [7]. Clinically, dPCs tend to have a more aggressive course – behaving similarly to Gleason 4 + 4 = 8 carcinomas [8]. Tumors with >10% ductal component are associated with a higher stage, are more likely to present with metastatic disease, and may be less responsive to androgen deprivation [7].

While the more aggressive clinical course associated with dPC has been well documented, little is known about the molecular features underlying this histologic subtype. Studies using fluorescence in situ hybridization have reported the prevalence of *TMPRSS2:ERG* fusions in ductal cases to range from approximately 10–50%, which is not substantially different than typical acinar carcinomas [9, 10]. Otherwise, gene expression profiling studies reveal extensive similarities between ductal and acinar adenocarcinomas. In one study comparing the transcriptional profile of eight ductal tumors to 11 acinar adenocarcinomas, differences in gene expression profiles encompassed only 25 genes [11].

Given that little is known regarding the underlying genomic abnormalities associated with the ductal histologic phenotype, we sequenced consecutive cases of dPC using the UW-OncoPlex platform – a targeted next-generation sequencing panel that includes genes with actionable or potentially actionable mutations [12].

RESULTS

Patient characteristics

From January 2015 to April 2016, ten consecutive patients with dPC were identified and their tumors were sequenced (Figure 1). The median age at diagnosis was 59 years (range, 40 to 73). Four (40%) patients had metastatic disease at the time of presentation. Additional details regarding the patients included in this study and their tumor samples are provided in Table 1.

Sequencing results

To characterize the molecular features of dPC, we sequenced 10 prostate cancers with prominent dPC components: nine samples from FFPE archival tissue (radical prostatectomy or prostate needle biopsy specimens), and one frozen tissue biopsy from a metastasis. Nine of 10 samples had sufficient material for UW-OncoPlex testing. The tumors from four (40%) patients had an alteration predicted to be pathogenic in one of the mismatch repair (MMR) genes (2 in *MSH2*, 1 in *MSH6* and 1 in *MLH1*), of which 3 (75%) had evidence of hypermutation associated with microsatellite instability (MSI). The 3 patients with hypermutated tumors had evidence of bi-allelic MMR mutation. Other genomic alterations common to prostate cancer were also detected, including alterations in genes involved in homologous recombination repair (i.e. *BRCA2*, *CHEK2*) ($N = 2$), androgen receptor (*AR*) ($N = 1$), *TMPRSS2:ERG* rearrangements ($N = 3$) and alteration in the PI3K/Akt/mTOR signaling pathway ($N = 5$) (Table 2).

Histopathology of hypermutated prostate cancer

To determine the histopathologic features of hypermutated prostate cancer, we reviewed the pathology of known hypermutated cases from the University of Washington rapid autopsy program. We previously reported 5 prostate cancer patients who participated in this program and were found to have hypermutated tumors with complex MMR gene alterations [13]. Since that publication, we have identified 3 additional hypermutated prostate cancer cases using similar methods. Of the now 8 hypermutated prostate cancer cases in the autopsy series, 2 had untreated primary prostate cancer tissue available for pathology review. Both of these cases had a ductal adenocarcinoma component. The first subject (Autopsy Patient: 05-165) was previously reported to have an *MSH2-C2orf61* 343 kb inversion, *MSH2-KCNK12* 74 kb inversion, and *MSH2-KCNK12* 40 kb inversion [13]. The second subject (Autopsy Patient: 01-002), who was not included in our previous publication, had a germline *MSH2* exon 1–8 deletion with loss of heterozygosity in tumor tissue.

The tumor of a third patient with known hypermutated prostate cancer (determined through previously described methods) being followed in our clinic was histologically reviewed [14]. There was no ductal adenocarcinoma component in his tumor. It is notable, however, that this patient had a PSA decline following treatment with the immune checkpoint inhibitor pembrolizumab (i.e. anti-PD1) despite previously progressing on abiraterone, enzalutamide, docetaxel, carboplatin and cabazitaxel (Figure 2).

DISCUSSION

This series of consecutive patients with dPC represents the largest next-generation sequencing study focused on this rare prostate cancer subset to date. Consistent with other published reports, patients in our series had aggressive clinical features, including young age at diagnosis and a high proportion of metastatic disease at presentation [6–8, 15]. Surprisingly, we found that alterations in MMR genes and associated hypermutation were far more prevalent in dPC compared to prostate cancers not selected by histologic subtype [13, 14]. Providing further support for an association between ductal histology and MMR deficiency, we found that

two of three patients with MMR-deficient hypermutated metastatic prostate cancer whose primary tumors were available for review had dPC.

Hypermutated prostate cancers have only recently been described, with initial reported incidence ranging from approximately 3% to 12% in men with metastatic castration-resistant prostate cancer (mCRPC) [13, 14]. Although further validation to establish prevalence through larger systematic studies is needed, our findings are intriguing because they suggest a potential histologic association between the hypermutated genotype and a ductal histopathologic phenotype. More broadly, this finding supports an argument for sequencing rare histologic subtypes, as histology may provide insights into a tumor's underlying molecular features. Indeed, it is notable that a similar genotype-phenotype correlation in hypermutated MSI colorectal cancer has also been described – lending credence to the possibility that hypermutated cancers may have distinct histology compared to matched microsatellite stable cases [1, 3].

Determining which patients have hypermutated prostate tumors may have important implications for future precision oncology trials, as mutational burden has been shown to correlate with response to immune checkpoint blockade in several tumor types (e.g. anti-CTLA4, anti-PD1, anti-PDL1) [16–18]. Although objective responses to immune checkpoint inhibition have initially been generally disappointing in patients with prostate cancer, most have a relatively low mutational load [19, 20]. A recent Phase II study testing pembrolizumab (anti-PD1 therapy) in patients with metastatic colorectal carcinoma with and without MMR deficiency reported that 40% of hypermutated colorectal cancer patients had an immune-related objective response (irOR) compared to 0% of patients without MSI-high tumors. Moreover, a 50% response rate to pembrolizumab in hypermutated non-colorectal gastrointestinal malignancies has been observed – supporting the hypothesis that mutational load may be a predictive biomarker for response to immune checkpoint blockade in prostate cancer [17]. The observation that one of the hypermutated patients followed in our clinic had a dramatic response to anti-PD1 therapy in spite of being heavily pretreated further bolsters the hypothesis that hypermutation may be predictive of response to PD1/PDL1 pathway inhibition.

Consistent with our prior observations, we found that somatic loss-of-function mutations in *MSH2* and *MSH6* were the primary cause of microsatellite instability in patients with prostate cancer [13].This is in contrast to colorectal cancer where hypermutation has been found to be associated with epigenetic silencing of *MLH1*, which occurs in nearly 2/3 of the cases [21]. Interestingly, the tumor of Subject #2 showed evidence of *MSH2* inversion without clear evidence of hypermutation or MSI. Whether the *MSH2* loss-of-function alteration represents an early event and hypermutation is a later consequence in the disease course or follows selective pressures of treatment will need to be further examined. Given that the mechanisms underlying prostate cancer hypermutation appear distinct from colorectal cancer, patterns of MSI may also be divergent and a tailored approach to MSI testing of prostate cancer may be needed. However, our findings suggest that ductal histology may be a cue to investigate further for evidence of MMR deficiency and hypermutation.

The finding that prostate cancers with ductal histologic features may be enriched for somatic hypermutation is intriguing; however, our small sample size limits our ability to draw definitive conclusions regarding this genotype-histologic phenotype relationship. If this finding is confirmed, however, the presence of ductal

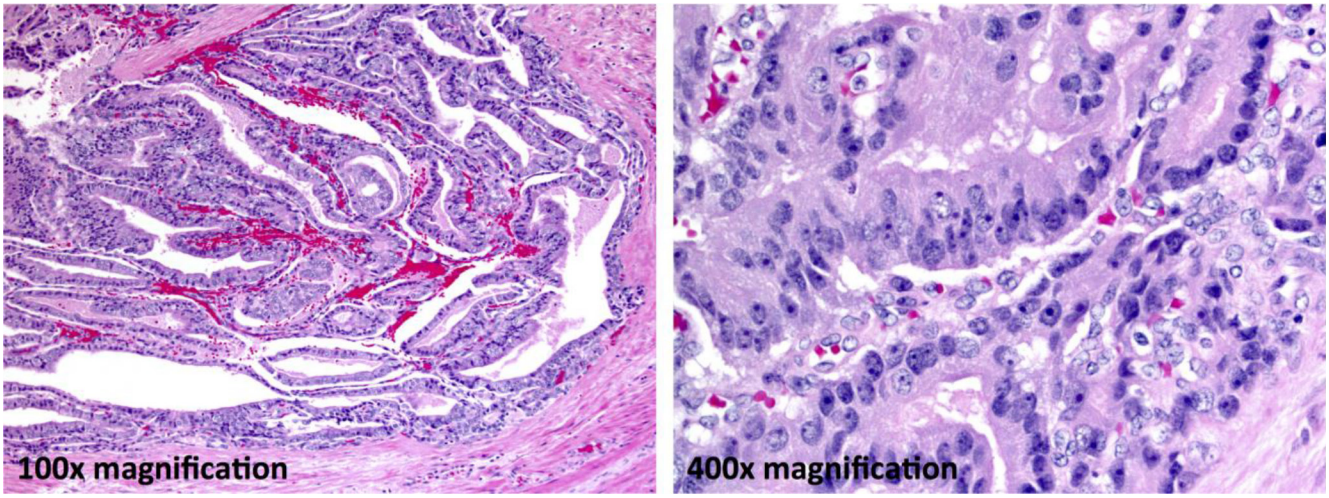


Figure 1: Ductal adenocarcinoma component. In this case, approximately 65% of the carcinoma is ductal. Large tumor cell aggregates have a tubulopapillary architecture (100× final magnification). Forming a pseudostratified columnar epithelium the tumor cells have markedly atypical nuclei with clumped chromatin and prominent nucleoli (400× final magnification).

Table 1: Demographics

Subject number	Age at diagnosis	Gleason	Disease state at presentation	Disease state at Time of Tissue Acquisition	Source of tissue for UW-OncoPlex	Clinical state at last follow up	Time from diagnosis to last follow up (months)
1	72	9	Localized	Localized	Prostatectomy	NED	34.8
2	69	9	Metastatic	mHSPC	Needle Biopsy	mHSPC	8.2
3	52	8	Localized	Localized	Prostatectomy	NED	16.6
4	66	9	Localized	Localized	Prostatectomy	Death	10.3
5	73	7	Localized	Localized	Prostatectomy	Biochemical recurrence	28.1
6	51	8	Localized	Localized	Prostatectomy	NED	28.7
7	40	9	Metastatic	mCRPC	Prostatectomy	mHSPC	1.0
8	61	9	Metastatic	mHSPC	Needle Biopsy	mHSPC	29.6
9	58	9	Localized	mHSPC	Needle Biopsy	NED	15.3
10	54	7	Metastatic	Localized	Soft Tissue Met	mCRPC	16.5

mHSPC, metastatic hormone-sensitive prostate cancer; NED, no evidence of disease; mCRPC, metastatic castration-resistant prostate cancer.

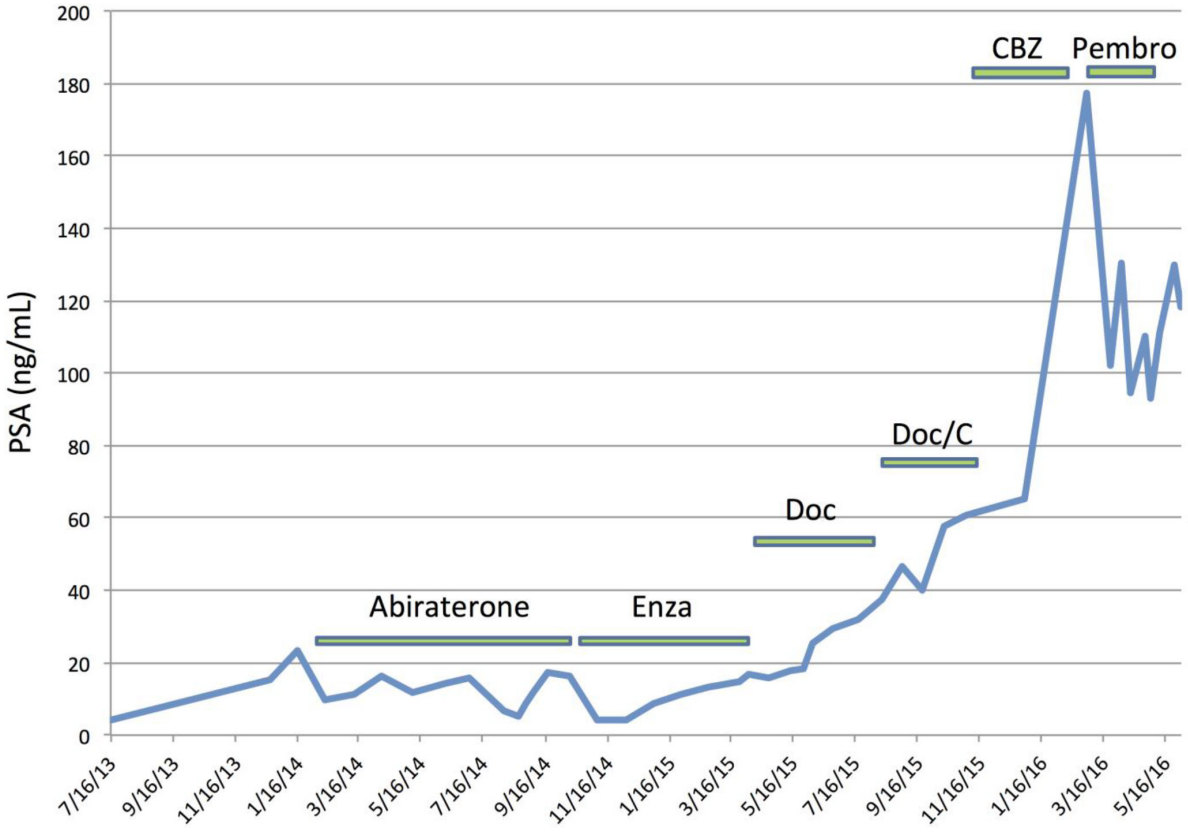


Figure 2: PSA response to checkpoint blockade immunotherapy in a patient with hypermutated prostate cancer. Prior to initiating pembrolizumab, this patient had bone, adrenal and lymph node metastases, and a baseline PSA of 177.35 ng/mL. A total of 3 cycles of pembrolizumab were administered before stopping due to an immune related adverse event (anasarca) requiring corticosteroids. He expired in June 2016. Note: this patient did not have ductal histopathologic features. Enza, enzalutamide; Doc, docetaxel; C, carboplatin; CBZ, cabazitaxel; Pembro, pembrolizumab.

Table 2: Summary of somatic alterations identified in ductal prostate cancer cases

Subject number	Ductal component of sample used for NGS	Tumor content estimated from NGS	MMR gene alteration	HR gene alteration	Hypermutated	Total Coding Mutations (per 1.2Mb sequenced)	Selected Other Mutations and Variants
1	71%	30%	No	*CHEK2* c.1100delC+LOH	No	4	*PIK3CA* p.H1047Y, *PIK3R1* p.R577del, *CDH1* p.P373L, *EPHA5* p.R896H
2	45%	40%	*MSH2* inversion	No	No	4	*TP53* p.L252_I254del, *FOXA1* p.S304R
3	65%	60%	No	No	No	4	*TMPRSS2:ERG* rearrangement, *PTEN* p.F90Lfs*9 (only in 4% of reads), IKZF1 p.E35K, ABL2 c.347-1G>T, PML p.V452M, and *TRRAP* p.E1229Q
4	30%	60%	*MSH6* c.1900_1901del+LOH	No	Yes	29	*PTEN c.*968dup
5	97%	50%	*MSH2-GRHL2* rearrangement +LOH	No	Yes	34	(Many frameshift mutations attributable to MSI)
6	99%	50%	No	No	No	5	*IDH1*p.R132C, *CTNNB1* (beta catenin) p.S33A, and *FOXA1* p.M253_F254del
7	25%	0%	–	–	–	–	Insufficient tissue for sequencing
8	31%	70%	No	No	No	5	*PTEN* copy loss, *TMPRSS2:ERG* rearrangement, *TP53* p.E258G
9	35%	10%	No	*BRCA2* c.5946delT+likely LOH	No	3	*SPOP* p.D130E, *FLT1* (VEGFR) rearrangement
10	-	60%	*MLH1* exon 19+ 3'UTR homozygous deletion	No	Yes	32	*AR* p.W742L, *PIK3CA* p.H1047R, *TMPRSS2:ERG* rearrangement, *FOXA1* rearrangement

All mismatch repair (MMR) gene and homologous recombination (HR) gene alterations were known or predicted to be pathogenic. Note: metastatic tissue from subject 10 was sequenced. LOH, loss of heterozygosity; NGS, next generation sequencing.

adenocarcinoma histology could be a means to prioritize patients for additional studies to assess mutational burden, which may have clinical implications, as hypermutation appears to predict for response to immune checkpoint blockade in several cancer types, including early signals in prostate cancer [22]. Future efforts to define the landscape of genomic alterations in patients with this prostate cancer variant will likely require multi-institutional studies. Such studies may facilitate the promise and rapid completion of precision oncology approaches for targeting this molecular subset of prostate cancer.

MATERIALS AND METHODS

Patients

All patients carried a diagnosis of prostate cancer and were followed by a medical oncologist at the University of Washington Medical Center or Seattle Cancer Care Alliance (both in Seattle, Washington). Consecutive patients with a component of ductal adenocarcinoma were identified by the treating medical oncologist and offered tumor sequencing. After obtaining written informed consent, tumor samples were tested on the UW-OncoPlex platform [12].The original diagnoses of dPC, made by genitourinary (GU) pathologists (M.S.T., F.V.L.), were independently verified by a third GU pathologist (L.T.).

Ethics statement

This study was performed in accordance with the declaration of Helsinki guidelines and with ethics approval from the Institutional Review Board at the Fred Hutchinson Cancer Research Center/University of Washington Comprehensive Cancer Consortium.

Macrodissection of tumor tissue

Hematoxylin and eosin stained sections of the tumors were reviewed by an anatomic and molecular pathologist. Ten-micron unstained recut sections were cut from the FFPE block, which were determined to contain the maximum amount of ductal adenocarcinoma. The dPC component, which ranged from 20% to 99% of the cells by visual estimate of each tumor, was macrodissected prior to deparaffinization and DNA extraction.

Next-generation sequencing (NGS) testing

DNA was extracted from FFPE samples as previously described [12]. Fresh tumor samples were snap frozen and unselected tissue was submitted for DNA extraction. UW-OncoPlex was performed according to previously published methods [12]. Microsatellite instability (MSI) testing was performed directly on NGS data using the mSINGS method [23]. Total mutation

burden was estimated from targeted NGS data as previously described, with hypermutation defined as > 12 mutations/megabase [24].

ACKNOWLEDGMENTS

We thank all the men who generously participated in this research. We are grateful to Agnes Gawne, Lori Kollath and Hiep Nguyen for their assistance in this work. We acknowledge funding support from PNW Prostate SPORE CA097186; DOD awards PC131820, W81XWH-15-1-0562 and W81XWH-15-1-0430; NCI Cancer Center Support Grant 5P30 CA015704-40; the Prostate Cancer Foundation (including Young Investigator Awards to MTS, HHC, and CCP); and an award from the FHCRC Solid Tumor Translational Research Program.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Bessa X, Alenda C, Paya A, Alvarez C, Iglesias M, Seoane A, Dedeu JM, Abuli A, Ilzarbe L, Navarro G, Pellise M, Balaguer F, Castellvi-Bel S, et al. Validation microsatellite path score in a population-based cohort of patients with colorectal cancer. J Clin Oncol. 2011; 29:3374–80. doi: 10.1200/jco.2010.34.3947.
2. Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood. 2008; 111:2505–15. doi: 10.1182/blood-2007-07-102798.
3. Shia J, Ellis NA, Paty PB, Nash GM, Qin J, Offit K, Zhang XM, Markowitz AJ, Nafa K, Guillem JG, Wong WD, Gerald WL, Klimstra DS. Value of histopathology in predicting microsatellite instability in hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer. Am J Surg Pathol. 2003; 27:1407–17.
4. Fenton PA, Clarke SE, Owen W, Hibbert J, Hodgson SV. Cribriform variant papillary thyroid cancer: a characteristic of familial adenomatous polyposis. Thyroid. 2001; 11:193–7. doi: 10.1089/105072501300042965.
5. Sainty D, Liso V, Cantu-Rajnoldi A, Head D, Mozziconacci MJ, Arnoulet C, Benattar L, Fenu S, Mancini M, Duchayne E, Mahon FX, Gutierrez N, Birg F, et al. A new morphologic classification system for acute promyelocytic leukemia distinguishes cases with underlying PLZF/RARA gene rearrangements. Blood. 2000; 96:1287–96.
6. (2016). WHO Classification of Tumoours of the Urinary System and Male Genital Organs: World Health Organization.
7. Humphrey PA. Histological variants of prostatic carcinoma and their significance. Histopathology. 2012; 60:59–74. doi: 10.1111/j.1365-2559.2011.04039.x.
8. Brinker DA, Potter SR, Epstein JI. Ductal adenocarcinoma of the prostate diagnosed on needle biopsy: correlation with clinical and radical prostatectomy findings and progression. Am J Surg Pathol. 1999; 23:1471–9.
9. Han B, Mehra R, Suleman K, Tomlins SA, Wang L, Singhal N, Linetzky KA, Palanisamy N, Zhou M, Chinnaiyan AM, Shah RB. Characterization of ETS gene aberrations in select histologic variants of prostate carcinoma. Mod Pathol. 2009; 22:1176–85. doi: 10.1038/modpathol.2009.79.
10. Morais CL, Herawi M, Toubaji A, Albadine R, Hicks J, Netto GJ, De Marzo AM, Epstein JI, Lotan TL. PTEN loss and ERG protein expression are infrequent in prostatic ductal adenocarcinomas and concurrent acinar carcinomas. Prostate. 2015; 75:1610–9. doi: 10.1002/pros.23042.
11. Sanati S, Watson MA, Salavaggione AL, Humphrey PA. Gene expression profiles of ductal versus acinar adenocarcinoma of the prostate. Mod Pathol. 2009; 22:1273–9. doi: 10.1038/modpathol.2009.103.
12. Pritchard CC, Salipante SJ, Koehler K, Smith C, Scroggins S, Wood B, Wu D, Lee MK, Dintzis S, Adey A, Liu Y, Eaton KD, Martins R, et al. Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. J Mol Diagn. 2014; 16:56–67. doi: 10.1016/j.jmoldx.2013.08.004.
13. Pritchard CC, Morrissey C, Kumar A, Zhang X, Smith C, Coleman I, Salipante SJ, Milbank J, Yu M, Grady WM, Tait JF, Corey E, Vessella RL, et al. Complex MSH2 and MSH6 mutations in hypermutated microsatellite unstable advanced prostate cancer. Nat Commun. 2014; 5:4988. doi: 10.1038/ncomms5988.
14. Robinson D, Van Allen EM, Wu YM, Schultz N, Lonigro RJ, Mosquera JM, Montgomery B, Taplin ME, Pritchard CC, Attard G, Beltran H, Abida W, Bradley RK, et al. Integrative clinical genomics of advanced prostate cancer. Cell. 2015; 161:1215–28. doi: 10.1016/j.cell.2015.05.001.
15. Morgan TM, Welty CJ, Vakar-Lopez F, Lin DW, Wright JL. Ductal adenocarcinoma of the prostate: increased mortality risk and decreased serum prostate specific antigen. J Urol. 2010; 184:2303–7. doi: 10.1016/j.juro.2010.08.017.
16. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS, Hollmann TJ, Bruggeman C, Kannan K, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med. 2014; 371:2189–99. doi: 10.1056/NEJMoa1406498.
17. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, Biedrzycki B, Donehower RC, Zaheer A, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med. 2015; 372:2509–20. doi: 10.1056/NEJMoa1500596.
18. Rosenberg JE, Hoffman-Censits J, Powles T, van der Heijden MS, Balar AV, Necchi A, Dawson N, O'Donnell PH,

Balmanoukian A, Loriot Y, Srinivas S, Retz MM, Grivas P, et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. Lancet. 2016; 387:1909–20. doi: 10.1016/s0140-6736(16)00561-4.

19. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012; 366:2443–54. doi: 10.1056/NEJMoa1200690.

20. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Jr., Kinzler KW. Cancer genome landscapes. Science. 2013; 339:1546–58. doi: 10.1126/science.1235122.

21. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012; 487:330–7. doi: 10.1038/nature11252.

22. Graff JN, Alumkal JJ, Drake CG, Thomas GV, Redmond WL, Farhad M, Cetnar JP, Ey FS, Bergan RC, Slottke R, Beer TM. Early evidence of anti-PD-1 activity in enzalutamide-resistant prostate cancer. Oncotarget. 2016; 7:52810–17. doi: 10.18632/oncotarget.10547.

23. Salipante SJ, Scroggins SM, Hampel HL, Turner EH, Pritchard CC. Microsatellite instability detection by next generation sequencing. Clin Chem. 2014; 60:1192–9. doi: 10.1373/clinchem.2014.223677.

24. Haraldsdottir S, Hampel H, Tomsic J, Frankel WL, Pearlman R, de la Chapelle A, Pritchard CC. Colon and endometrial cancers with mismatch repair deficiency can arise from somatic, rather than germline, mutations. Gastroenterology. 2014; 147:1308–16.e1. doi: 10.1053/j.gastro.2014.08.041.

Clinical Commentary

Prostate Cancer Screening in a New Era of Genetics

Heather H. Cheng,[1,2] Colin C. Pritchard,[3] Bruce Montgomery,[1] Daniel W. Lin,[2,4] Peter S. Nelson[1,2]

Abstract

Men who inherit pathogenic germline mutations in *BRCA2* and *BRCA1* are at increased risk of developing aggressive prostate cancer, and those with germline mutations in other DNA repair genes such as *ATM, CHEK2, and MSH2/MSH6* may also have increased risks. Although clinically important, there is lack of specific guidance regarding management strategies for men at increased risk owing to germline mutation status or family history of aggressive prostate cancer. We review prostate cancer genetic risk factors and the ongoing IMPACT (Identification of Men with a genetic predisposition to ProstAte Cancer: Targeted screening in *BRCA1/2* mutation carriers and controls) screening study. Pending results of IMPACT and unified guidelines, there are areas of uncertainty and need for further study. Ongoing and future research will be critical for optimizing prostate cancer screening approaches for men at the highest risk for aggressive prostate cancer. In the interim, we propose a practical approach to prostate cancer screening for men with a germline mutation in a known/suspected moderate to high-penetrance cancer predisposition gene (eg, *BRCA1/2*), and/or men with a first- or second-degree relative with metastatic prostate cancer (regardless of genetic testing): baseline prostate-specific antigen and digital rectal exam by experienced providers at age 40 years or 5 years earlier than age of diagnosis of the youngest first- or second-degree relative with metastatic prostate cancer, whichever is earlier. Then, based on age, digital rectal exam, and prostate-specific antigen, we suggest consideration of magnetic resonance imaging, biopsy, and/or continued monitoring.

Clinical Genitourinary Cancer, Vol. ■, No. ■, ■-■ © 2017 Elsevier Inc. All rights reserved.
Keywords: BRCA, Early detection, Germline, Prostate, Screening

The past several years have delivered a succession of notable discoveries in prostate cancer involving DNA repair genes (DRGs) that have important implications for clinical care. These findings include somatic loss of DRG function in 20% of metastatic prostate cancers, high rates of therapeutic responses to PARP inhibitors and platinum chemotherapy in those tumors with homologous recombination DNA repair defects, and far higher than expected germline DRG mutations in men with metastatic prostate cancer.[1,2] In 2016, a dedicated study of nearly 700 men with metastatic prostate cancer found that 11.8% carried presumed pathogenic germline mutations in DRGs associated with cancer predisposition,[3] and subsequent studies have confirmed similarly high rates in metastatic compared with localized prostate cancer cases.[4-6] These changes point to new opportunities for precision oncology in prostate cancer, but also bring new conundrums. Germline genetic testing (ideally with genetic counseling support) to identify those men with advanced prostate cancer who harbor somatic homologous recombination DNA repair deficiency is already beginning to occur, as these individuals may benefit from PARP inhibitors and platinum chemotherapy. In contrast to guidelines for female carriers of *BRCA1/2* mutations at risk for breast and ovarian cancers, there is a lack of consensus for how to manage the male relatives including the brothers, sons, and nephews who undergo cascade testing and are found to carry the same mutation. How should we counsel and manage an unaffected man who carries a germline pathogenic *BRCA2* mutation? More challenging, what about a man with a pathogenic mutation in a newly implicated gene such as *ATM*, *CHEK2*, *or PALB2*, or a variant of uncertain significance (VUS)?

To begin to address these important issues, a brief review of prostate cancer genetic risk is warranted. Prostate cancer is one of the most heritable cancers, and family history of prostate cancer is a well-established risk factor.[7,8] An updated analysis of the Nordic Twin Studies estimates that up to 57% of prostate cancer risk may

[1]Department of Medicine, University of Washington, Seattle, WA
[2]Fred Hutchinson Cancer Research Center, Seattle, WA
[3]Department of Laboratory Medicine, University of Washington, Seattle, WA
[4]Department of Urology, University of Washington, Seattle, WA

Submitted: Apr 21, 2017; Revised: May 17, 2017; Accepted: May 23, 2017

Address for correspondence: Heather H. Cheng, MD, PhD, Division of Medical Oncology, University of Washington, Seattle Cancer Care Alliance, 825 Eastlake Ave E, Seattle, WA 98109
E-mail contact: hhcheng@uw.edu

1558-7673/$ - see frontmatter © 2017 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.clgc.2017.05.024

be accounted for by inherited factors.[4,9,10] These factors are comprised of 2 classes: (1) common variants (single nucleotide polymorphisms) identified through genome-wide association studies that individually carry a slightly increased risk, and (2) rare variants or mutations in genes that confer substantially higher risk if altered (eg, *BRCA1/2*). Genome-wide association studies have largely dominated prostate cancer research for the past few decades, with over 100 loci (eg, 8q24, 17p) implicated that may collectively account for up to one-third of familial risk of prostate cancer. However, these single nucleotide polymorphisms have not yet been incorporated into clinical practice, in part owing to relatively modest effects on risk (eg, less than 2-fold increases).[11,12]

On a population basis, relatively rare pathogenic germline mutations in tumor suppressor genes disrupt critical gene function and result in a significantly elevated risk of developing certain cancers. Pathogenic germline mutations in *BRCA2* and *BRCA1*, for example, have been studied in association with autosomal dominant hereditary breast and ovarian cancer predisposition syndrome. Studies have now shown that mutations in these genes also confer increased risks of developing prostate cancer, and more importantly, these cancers behave aggressively with higher rates of disease recurrence after primary treatment and increased mortality. Consequently, we recommend that men in families with relatives found to have a pathogenic *BRCA1* or *BRCA2* mutation also have germline testing. We strongly recommend consulting with a genetics professional when possible, especially when considering/planning cascade testing of family members (generally recommended once individuals are over the age of 18 years). This recommendation stems from potential patient and family confusion and stress around genetic testing results that may be delivered without appropriate pre- and post-test counseling. Many tests issue the following categories of result: pathogenic, likely pathogenic, uncertain significance, likely benign, and benign, with "likely" used to mean a greater than 90% certainty of a variant being disease-causing or benign with specific criteria to evaluate supporting evidence.[13] Uncertain significance is ascribed when neither criteria for pathogenic/likely pathogenic or benign/likely benign are met. In the case of variants of uncertain significance, we recommend considering clinical risk factors, such as family history, to guide management.

Pathogenic germline mutations in *BRCA1* and *BRCA2* are estimated to confer 1.1- to 3.8-fold[4,14,15] and 4.7- to 8.6-fold increased risks of prostate cancer, respectively.[11,16,17] Moreover, a growing body of data indicates that men with prostate cancer who carry germline pathogenic *BRCA2* mutations have earlier onset disease and worse prostate cancer outcomes and survival.[5,18-20] The evidence for germline pathogenic *BRCA1* mutation carriers is less clear, though *BRCA1* mutations have been observed at a higher rate in the metastatic setting, suggesting a similar association. Thus, there is rationale for considering men who carry pathogenic and likely pathogenic mutations in high penetrance germline cancer predisposition genes as a group likely to be at particularly high risk for developing aggressive prostate cancer.

Currently there is a lack of consensus and specific direction in the prostate cancer screening and early detection guidelines in many of the professional societies, including the American Urological Association (2013),[21] National Comprehensive Cancer Network (2016),[22] and American Cancer Society (2016).[23] The current draft prostate cancer screening guidelines from the United States Preventative Service Task Force recommend that men with a family history of prostate cancer talk to their clinician about the potential benefits and harms of screening, with no additional specific guidance for *BRCA1/2* mutation carriers.[24] The National Comprehensive Cancer Network guidelines suggest inquiring about family history of *BRCA1/2* mutations, but stop short of further recommendations.[6,22] This is likely the result of guideline committees calling for evidence that is incomplete or pending with respect to prostate cancer screening in *BRCA1/2* mutation carriers.

The ongoing international IMPACT (Identification of Men with a genetic predisposition to ProstAte Cancer: Targeted screening in *BRCA1/2* mutation carriers and controls) study was designed to assess a targeted screening approach for mutation carriers and non-carriers as controls (clinicaltrials.gov; NCT00261456).[25] The initial screening round used a strategy of annual prostate-specific antigen (PSA) measurements followed by prostate biopsy for PSA $>$ 3.0 ng/mL. The positive predictive value for biopsy was higher in *BRCA2* mutation carriers compared with non-carriers (48% vs. 33%), and a significant difference was observed in detecting intermediate- or high-risk disease (68% vs. 43%) even within the first year of the study. Similarly, the positive predictive value for biopsy was higher in *BRCA1* mutation carriers compared with non-carriers (41% vs. 23%), although a significant difference in detecting intermediate- or high-risk disease were not observed within the first year. Longer follow-up and final results are eagerly anticipated, but even after completion, there will be unanswered questions.

In the case of known germline pathogenic *BRCA1/2* carriers for whom prostate cancer risk estimates are described, the biopsy threshold of PSA $>$ 3.0 ng/mL used in IMPACT can likely be further refined, even if it is demonstrated to be useful. For example, we will not learn from the IMPACT study if PSA $>$ 3.0 ng/mL is the optimal threshold to trigger biopsy. An alternative approach is to use a PSA threshold to recommend biopsy if the PSA exceeds the age-specific mean (which can be substantially lower than 4.0 ng/mL or the 3.0 ng/mL used for IMPACT). This approach may be complicated by lack of standard age-specific thresholds as well as by differences based on genetic background.[26-29] One series reported that lowering the cutpoint to $>$ 2.5 ng/mL may have a favorable detection rate with lower rates of eventual PSA progression.[30] In the Prostate Cancer Prevention Trial, high-grade prostate cancers were found in 12.5% of men with PSA $<$ 0.5 ng/mL.[31] This suggests that even a very low PSA threshold will miss some aggressive cancers and may justify considering a biopsy regardless of PSA if there is evidence of abnormality by another measure such as imaging or digital rectal exam (DRE).

The utility of early detection strategies for any cancer depends on a predictable natural history and disease course, typically on the order of years to decades. Colorectal cancer screening strategies are successful because adenocarcinomas typically arise from precancerous polyps on a temporal scale of many years, during which polyps can be detected and removed before or at least early in the disease course. By comparison, pancreatic cancer is much more challenging to detect early and effectively intervene upon owing to a relatively compressed temporal scale, conceivably on the order of months to years. It is tempting to take what we know about the increased aggressiveness of *BRCA1/2*-associated prostate cancer and conclude

that we should simply start screening earlier and more frequently in men who are at increased genetic risk. It is possible that this approach is correct, but if the natural history of *BRCA1/2*-associated prostate cancer resembles pancreatic cancer more than it does colorectal cancer, then early screening alone may not be the best approach.[32] We need more data. Our collective understanding of the natural history and disease pace of *BRCA1/2*-associated prostate cancer is incomplete, and the existing data is subject to ascertainment and other sources of bias.

Consider also the "long tail" of new DNA repair genes observed to be altered in the germline of men with metastatic disease that are now candidate prostate cancer risk genes: *ATM*, *CHEK2*, *PALB2*, *RAD51C*, and *RAD51D*, etc. We know even less about these genes compared with *BRCA1/2* and have little to no risk estimates for carriers. For women who carry mutations in *ATM*, *CHEK2*, *PALB2*, *RAD51C*, and *RAD51D*, for example, better data about relative risk and penetrance of individual genes have led to gene-specific cancer screening guidelines.[33] Although disease manifestation and organ specificity will likely be different for prostate cancer, reference to breast and ovarian cancer literature may still be of value pending more prostate cancer-specific data. There may also be as-yet-undiscovered genes and mechanisms of inherited prostate cancer predisposition that will be missed by current testing methods. Thus, men with a strong family history of aggressive prostate cancer should still be considered at increased risk, even if genetic testing results do not clearly identify a familial cancer predisposition.

How do we move forward with prostate cancer screening in this new era of genetics? As researchers, it is our task to design studies and gather data that will refine and improve care for future patients. At the same time, as physicians, we must also be pragmatists in the face of incomplete evidence and care for the patients who sit in front of us today as responsibly as we can. Given the lack of clarity about prostate cancer screening for high-risk men, we propose the following approach while eagerly awaiting more data to support and refine specific strategies that reflect benefit and harm.

Proposed Prostate Cancer Screening Approach for Germline Carriers of *BRCA1/2* Mutations[a]

For men who test positive for a pathogenic or likely pathogenic variant in a high-penetrance germline cancer predisposition gene (eg, *BRCA1/2*)[a] and/or who have a first- or second-degree relative with metastatic prostate cancer (regardless of genetic testing):

- Baseline PSA and DRE by an experienced provider at age 40 years or 5 years earlier than the age of diagnosis of the youngest first- or second-degree relative with metastatic prostate cancer, whichever is earlier.
- If DRE is abnormal, suggest biopsy, regardless of PSA.
- If DRE is normal, then follow the chart in Table 1.

Participation in prostate cancer screening trials/programs such as IMPACT should be encouraged whenever available. To the extent possible, completed studies should be reevaluated with germline analysis and consider high-risk groups separately. If enrollment in a screening trial is not possible, men with germline mutations in *BRCA1/2*, along with other DNA repair mutations such as *ATM*, *CHEK2*, *PALB2*, *RAD51C*, etc. should be encouraged to participate in patient registries where pre-diagnostic PSAs, biopsies, imaging and clinical outcomes, etc. can be collected and analyzed with new statistics and modeling tools to further hone early detection strategies.[34,35] For women who carry *BRCA1/2* mutations, models have been developed to guide decisions between multiple screening and prevention strategies.[36] It is our hope that guidelines for men who carry germline cancer predisposition genes will expand and someday soon include similarly tailored prostate cancer screening strategies.

Table 1 Suggested PSA (ng/mL) Thresholds for Action in Germline Carriers of *BRCA1/2* Mutations[b]

	Age, y[a]		
	≤49	50-59	60-69
Repeat PSA and DRE yearly	<0.75	<1.0	<1.5
Consider biopsy, biomarker(s) and/or MRI. Repeat PSA/DRE yearly	0.75-1.5	1.0-2.0	1.5-2.5
Suggest biopsy with/without MRI	>1.5	>2.0	>2.5

Abbreviations: DRE = digital rectal exam; MRI = magnetic resonance imaging; PSA = prostate-specific antigen.
[a]PSA thresholds set at/above upper limit of age-matched population ranges reported in published series.[4,9,11]
[b]Consultation at a center of excellence and clinical trial/registry participation encouraged whenever possible.

We are at the cusp of a new era of cancer genetics. The new developments have already begun to make a positive difference in the lives of men with advanced prostate cancer. Attention to how we might similarly adjust the way we think about prostate cancer screening and early detection for the brothers, sons, nephews, and cousins who are at high risk for aggressive disease looks to a future where we might truly avert prostate cancer deaths.

Acknowledgments

The authors gratefully acknowledge support from the Prostate Cancer Foundation, including Young Investigator Awards (to H.H.C. and C.C.P.), Congressional Designated Medical Research Program (CDMRP) award PC131820 (C.C.P.), the Institute for Prostate Cancer Research, and the Pacific Northwest Prostate Cancer SPORE CA097186.

The views expressed here are our own and not an official position of our institutions or funders.

Disclosure

The authors have stated that they have no conflicts of interest.

References

1. Mateo J, Carreira S, Sandhu S, et al. DNA-repair defects and olaparib in metastatic prostate cancer. *N Engl J Med* 2015; 373:1697-708.
2. Cheng HH, Pritchard CC, Boyd T, et al. Biallelic inactivation of BRCA2 in platinum-sensitive metastatic castration-resistant prostate cancer. *Eur Urol* 2016; 69:992-5.

[a] This approach can also be considered for men who carry a pathogenic or likely pathogenic variant of suspected moderate penetrance prostate cancer risk genes associated with aggressive disease (eg, *ATM*, *CHEK2*, etc.[3]), and/or who have a strong family history of prostate and other cancers.

3. Pritchard CC, Mateo J, Walsh MF, et al. Inherited DNA-repair gene mutations in men with metastatic prostate cancer. *N Engl J Med* 2016; 375:443-53.
4. Loeb S, Carter HB, Catalona WJ, et al. Baseline prostate-specific antigen testing at a young age. *Eur Urol* 2012; 61:1-7.
5. Na R, Zheng SL, Han M, et al. Germline mutations in ATM and BRCA1/2 distinguish risk for lethal and indolent prostate cancer and are associated with early age at death. *Eur Urol* 2017; 71:740-7.
6. Annala M, Struss WJ, Warner EW, et al. Treatment outcomes and tumor loss of heterozygosity in germline DNA repair-deficient prostate cancer. *Eur Urol* 2017; 72:34-42.
7. Bratt O, Drevin L, Akre O, et al. Family history and probability of prostate cancer, differentiated by risk category: a nationwide population-based study. *J Natl Cancer Inst* 2016; 108.
8. Hemminki K, Ji J, Forsti A, et al. Concordance of survival in family members with prostate cancer. *J Clin Oncol* 2008; 26:1705-9.
9. Tang P, Sun L, Uhlman MA, et al. Initial prostate specific antigen 1.5 ng/ml or greater in men 50 years old or younger predicts higher prostate cancer risk. *J Urol* 2010; 183:946-50.
10. Mucci LA, Hjelmborg JB, Harris JR, et al. Familial risk and heritability of cancer among twins in Nordic countries. *JAMA* 2016; 315:68-76.
11. Vickers AJ, Ulmert D, Sjoberg DD, et al. Strategy for detection of prostate cancer based on relation between prostate specific antigen at age 40-55 and long term risk of metastasis: case-control study. *BMJ* 2013; 346:f2023.
12. Eeles R, Goh C, Castro E, et al. The genetic epidemiology of prostate cancer and its clinical implications. *Nat Rev Urol* 2014; 11:18-31.
13. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015; 17:405-24.
14. Thompson D, Easton DF, Breast Cancer Linkage Consortium. Cancer incidence in BRCA1 mutation carriers. *J Natl Cancer Inst* 2002; 94:1358-65.
15. Leongamornlert D, Mahmud N, Tymrakiewicz M, et al. Germline BRCA1 mutations increase prostate cancer risk. *Br J Cancer* 2012; 106:1697-701.
16. Breast Cancer Linkage Consortium. Cancer risks in BRCA2 mutation carriers. *J Natl Cancer Inst* 1999; 91:1310-6.
17. Kote-Jarai Z, Leongamornlert D, Saunders E, et al. BRCA2 is a moderate penetrance gene contributing to young-onset prostate cancer: implications for genetic testing in prostate cancer patients. *Br J Cancer* 2011; 105:1230-4.
18. Castro E, Goh C, Leongamornlert D, et al. Effect of BRCA mutations on metastatic relapse and cause-specific survival after radical treatment for localised prostate cancer. *Eur Urol* 2015; 68:186-93.
19. Castro E, Goh C, Olmos D, et al. Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer. *J Clin Oncol* 2013; 31:1748-57.
20. Gallagher DJ, Gaudet MM, Pal P, et al. Germline BRCA mutations denote a clinicopathologic subset of prostate cancer. *Clin Cancer Res* 2010; 16:2115-21.
21. Carter HB, Albertsen PC, Barry MJ, et al. Early detection of prostate cancer: AUA Guideline. *J Urol* 2013; 190:419-26.
22. Carroll PR, Parsons JK, Andriole G, et al. NCCN Guidelines Insights: Prostate Cancer Early Detection, Version 2.2016. *J Natl Compr Canc Netw* 2016; 14:509-19.
23. Smith RA, Andrews K, Brooks D, et al. Cancer screening in the United States, 2016: a review of current American Cancer Society guidelines and current issues in cancer screening. *CA Cancer J Clin* 2016; 66:96-114.
24. US Preventive Services Task Force (USPSTF), Screening for Prostate Cancer, Available at: http://www.screeningforprostatecancer.org, Accessed July 6, 2017.
25. Bancroft EK, Page EC, Castro E, et al. Targeted prostate cancer screening in BRCA1 and BRCA2 mutation carriers: results from the initial screening round of the IMPACT study. *Eur Urol* 2014; 66:489-99.
26. Oesterling JE, Jacobsen SJ, Chute CG, et al. Serum prostate-specific antigen in a community-based population of healthy men. Establishment of age-specific reference ranges. *JAMA* 1993; 270:860-4.
27. Crawford ED. Prostate Cancer Awareness Week: September 22 to 28, 1997. *CA Cancer J Clin* 1997; 47:288-96.
28. Morgan TO, Jacobsen SJ, McCarthy WF, et al. Age-specific reference ranges for serum prostate-specific antigen in black men. *N Engl J Med* 1996; 335:304-10.
29. He D, Wang M, Chen X, et al. Ethnic differences in distribution of serum prostate-specific antigen: a study in a healthy Chinese male population. *Urology* 2004; 63:722-6.
30. Zhu H, Roehl KA, Antenor JA, et al. Biopsy of men with PSA level of 2.6 to 4.0 ng/mL associated with favorable pathologic features and PSA progression rate: a preliminary analysis. *Urology* 2005; 66:547-51.
31. Thompson IM, Pauler DK, Goodman PJ, et al. Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. *N Engl J Med* 2004; 350:2239-46.
32. Gulati R, Cheng HH, Lange PH, et al. Screening men at increased risk for prostate cancer diagnosis: model estimates of benefits and harms. *Cancer Epidemiol Biomarkers Prev* 2017; 26:222-7.
33. National Comprehensive Cancer Network, Genetic/Familial High-Risk Assessment: Breast and Ovarian (Version 2.2017), Available at: https://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf, Accessed July 6, 2017.
34. Gulati R, Gore JL, Etzioni R. Comparative effectiveness of alternative prostate-specific antigen–based prostate cancer screening strategies: model estimates of potential benefits and harms. *Ann Intern Med* 2013; 158:145-53.
35. Birnbaum JK, Feng Z, Gulati R, et al. Projecting benefits and harms of novel cancer screening biomarkers: a study of PCA3 and prostate cancer. *Cancer Epidemiol Biomarkers Prev* 2015; 24:677-82.
36. Kurian AW, Munoz DF, Rust P, et al. Online tool to guide decisions for BRCA1/2 mutation carriers. *J Clin Oncol* 2012; 30:497-506.

available at www.sciencedirect.com
journal homepage: www.europeanurology.com

European Association of Urology

Platinum Priority – Prostate Cancer
Editorial by XXX on pp. x–y of this issue

Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017

Silke Gillessen [a,], Gerhardt Attard [b], Tomasz M. Beer [c], Himisha Beltran [d], Alberto Bossi [e], Rob Bristow [f], Brett Carver [g], Daniel Castellano [h], Byung Ha Chung [i], Noel Clarke [j], Gedske Daugaard [k], Ian D. Davis [l], Johann de Bono [b], Rodolfo Borges dos Reis [m], Charles G. Drake [n], Ros Eeles [o], Eleni Efstathiou [p], Christopher P. Evans [q], Stefano Fanti [r], Felix Feng [s], Karim Fizazi [t], Mark Frydenberg [u], Martin Gleave [v], Susan Halabi [w], Axel Heidenreich [x], Celestia S. Higano [y], Nicolas James [z], Philip Kantoff [aa], Pirkko-Liisa Kellokumpu-Lehtinen [bb], Raja B. Khauli [cc], Gero Kramer [dd], Chris Logothetis [ee], Fernando Maluf [ff], Alicia K. Morgans [gg], Michael J. Morris [hh], Nicolas Mottet [ii], Vedang Murthy [jj], William Oh [kk], Piet Ost [ll], Anwar R. Padhani [mm], Chris Parker [nn], Colin C. Pritchard [oo], Mack Roach [s], Mark A. Rubin [pp], Charles Ryan [qq], Fred Saad [rr], Oliver Sartor [ss], Howard Scher [tt], Avishay Sella [uu], Neal Shore [vv], Matthew Smith [ww], Howard Soule [xx], Cora N. Sternberg [yy], Hiroyoshi Suzuki [zz], Christopher Sweeney [aaa], Matthew R. Sydes [bbb], Ian Tannock [ccc], Bertrand Tombal [ddd], Riccardo Valdagni [eee], Thomas Wiegel [fff], Aurelius Omlin [a]*

[a] *Department of Medical Oncology, Cantonal Hospital St. Gallen and University of Berne, Switzerland;* [b] *Department of Medical Oncology, The Institute of Cancer Research/Royal Marsden, London, UK;* [c] *Oregon Health & Science University Knight Cancer Institute, OR, USA;* [d] *Department of Medical Oncology, Weill Cornell Medicine, New York, NY, USA;* [e] *Department of Radiation Oncology, Genito Urinary Oncology, Prostate Brachytherapy Unit, Goustave Roussy, Paris, France;* [f] *Department of Radiation Oncology, Princess Margaret Cancer Centre and University of Toronto, Toronto, ON, USA;* [g] *Department of Urology, Sidney Kimmel Center for Prostate and Urologic Cancers, New York, NY, USA;* [h] *Department of Medical Oncology, Hospital Universitario 12 de Octubre, Madrid, Spain;* [i] *Department of Urology, Gangnam Severance Hospital, Yonsei University Health System, Seoul, Korea;* [j] *Department of Urology, The Christie and Salford Royal Hospitals, Manchester, UK;* [k] *Department of Medical Oncology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark;* [l] *Monash University and Eastern Health, Eastern Health Clinical School, Box Hill, Australia;* [m] *Department of Urology, Ribeirão Preto Medical School, University of São Paulo, São Paulo, Brazil;* [n] *Department of Medical Oncology, Division of Haematology/Oncology, Columbia University Medical Center, New York, NY, USA;* [o] *Department of Clinical Oncology and Genetics, The Institute of Cancer Research and Royal Marsden NHS Foundation Trust, London, UK;* [p] *Department of Medical Oncology, University of Texas MD Anderson Cancer Center, TX, USA;* [q] *Department of Urology, University of California, Davis School of Medicine, CA, USA;* [r] *Department of Nuclear Medicine, Policlinico S. Orsola, Università di Bologna, Italy;* [s] *Department of Radiation Oncology, University of California, San Francisco, CA, USA;* [t] *Department of Medical Oncology, Gustave Roussy, University of Paris Sud, Paris, France;* [u] *Department of Surgery, Department of Anatomy and Developmental Biology, Faculty of Medicine, Nursing and Health Sciences, Monash University;* [v] *Department of Urology, Vancouver Prostate Centre, University of British Columbia, Vancouver, BC, Canada;* [w] *Department of Clinical trials and Statistics, Duke University, Durham, NC, USA;* [x] *Department of Urology, University Hospital Köln, Köln, Germany;* [y] *Department of Medicine, Division of Medical Oncology, University of Washington and Fred Hutchinson Cancer Research Center, WA, USA;* [z] *Department of Clinical Oncology, Clinical Oncology Queen Elizabeth Hospital Birmingham and University of Birmingham, Birmingham, UK;* [aa] *Department of Medical Oncology, Memorial Sloan Kettering Cancer Center and Weill Cornell Medical College, New York, NY, USA;* [bb] *Department of Clinical Oncology, Tampere University Hospital, Faculty of Medicine and Life Sciences, University of Tampere, Finland;* [cc] *Department of Urology, American University of Beirut Medical Center, Beirut, Lebanon;* [dd] *Department of Urology, Medical University of*

[*] Corresponding author. Cantonal Hospital St. Gallen, Rorschacherstrasse 95, 9007 St. Gallen, Switzerland. Tel. +41 71 494 11 11; Fax: +41 71 494 63 25.
E-mail address: silke.gillessen@kssg.ch (S. Gillessen).

http://dx.doi.org/10.1016/j.eururo.2017.06.002
0302-2838/© 2017 European Association of Urology. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

Vienna, Vienna, Austria; [ee] *Department of Genitourinary Medical Oncology, MD Anderson Cancer Centre, Houston, TX, USA;* [ff] *Department of Medical Oncology Hospital Israelita Albert Einstein and Department of Medical Oncology Beneficência Portuguesa de São Paulo;* [gg] *Department of Medical Oncology and Epidemiology, Vanderbilt University Medical Center, Division of Hematology/Oncology, Nashville, TN, USA;* [hh] *Department of Medical Oncology, Memorial Sloan Kettering Cancer Center, New York, NY, USA;* [ii] *Department of Urology, University Hospital Nord St. Etienne, St. Etienne, France;* [jj] *Department of Radiation Oncology, Tata Memorial Centre, Mumbai, India;* [kk] *Department of Medical Oncology, Division of Hematology and Medical Oncology, Icahn School of Medicine at Mount Sinai, The Tisch Cancer Institute, New York, NY, USA;* [ll] *Department of Radiation Oncology, Ghent University Hospital, Ghent, Belgium;* [mm] *Department of Radiology, Mount Vernon Cancer Centre and Institute of Cancer Research, London, UK;* [nn] *Department of Clinical Oncology, Royal Marsden NHS Foundation Trust, Sutton, UK;* [oo] *Department of Pathology, University of Washington, WA, USA;* [pp] *Department of Pathology, University of Bern and the Inselspital, Bern (CH);* [qq] *Department of Medical Oncology, Clinical Medicine and Urology at the Helen Diller Family Comprehensive Cancer Center at the University of, California, San Francisco, CA, USA;* [rr] *Department of Urology, Centre Hospitalier de l'Université de Montréal, Montreal, QC, Canada;* [ss] *Department of Medical Oncology, Tulane Cancer Center, New Orleans, LA, USA;* [tt] *Department of Medical Oncology, Genitourinary Oncology Service, Memorial Sloan Kettering Cancer Centre, New York, NY, USA;* [uu] *Department of Medical Oncology, Department of Oncology, Assaf Harofeh Medical Centre, Tel-Aviv University, Sackler School of Medicine, Zerifin, Israel;* [vv] *Department of Urology, Carolina Urologic Research Center, Myrtle Beach, SC, USA;* [ww] *Department of Medical Oncology, Massachusetts General Hospital Cancer Centre, Boston, MA, USA;* [xx] *Prostate Cancer Foundation, Santa Monica, CA, USA;* [yy] *Department of Medical Oncology, San Camillo Forlanini Hospital, Rome, Italy;* [zz] *Department of Urology, Toho University Sakura Medical Center, Japan;* [aaa] *Department of Medical Oncology, Dana-Farber Cancer Institute and Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA;* [bbb] *MRC Clinical Trials Unit at UCL, Institute of Clinical Trials and Methodology, University College London, London, UK;* [ccc] *Department of Medical Oncology, Princess Margaret Cancer Centre and University of Toronto, Toronto, ON, Canada;* [ddd] *Department of Urology, Cliniques Universitaires Saint Luc, Brussels, Belgium;* [eee] *Department of Oncology and Haemato-oncology, Università degli Studi di Milano. Radiation Oncology 1, Prostate Cancer Program, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy;* [fff] *Department of Radiation Oncology, Klinik für Strahlentherapie und Radioonkologie des Universitätsklinikum Ulm, Albert-Einstein-Allee, Ulm, Germany*

Article info

Article history:
Accepted June 1, 2017

Associate Editor:
James Catto

Keywords:
Advanced and high-risk localized prostate cancer
Castration-naive and castration-resistant prostate cancer
Therapeutics
Consensus
Oligometastatic prostate cancer

Abstract

Background: In advanced prostate cancer (APC), successful drug development as well as advances in imaging and molecular characterisation have resulted in multiple areas where there is lack of evidence or low level of evidence. The Advanced Prostate Cancer Consensus Conference (APCCC) 2017 addressed some of these topics.
Objective: To present the report of APCCC 2017.
Design, setting, and participants: Ten important areas of controversy in APC management were identified: high-risk localised and locally advanced prostate cancer; "oligometastatic" prostate cancer; castration-naïve and castration-resistant prostate cancer; the role of imaging in APC; osteoclast-targeted therapy; molecular characterisation of blood and tissue; genetic counselling/testing; side effects of systemic treatment(s); global access to prostate cancer drugs. A panel of 60 international prostate cancer experts developed the program and the consensus questions.
Outcome measurements and statistical analysis: The panel voted publicly but anonymously on 150 predefined questions, which have been developed following a modified Delphi process.
Results and limitations: Voting is based on panellist opinion, and thus is not based on a standard literature review or meta-analysis. The outcomes of the voting had varying degrees of support, as reflected in the wording of this article, as well as in the detailed voting results recorded in Supplementary data.
Conclusions: The presented expert voting results can be used for support in areas of management of men with APC where there is no high-level evidence, but individualised treatment decisions should as always be based on all of the data available, including disease extent and location, prior therapies regardless of type, host factors including comorbidities, as well as patient preferences, current and emerging evidence, and logistical and economic constraints. Inclusion of men with APC in clinical trials should be strongly encouraged. Importantly, APCCC 2017 again identified important areas in need of trials specifically designed to address them.
Patient summary: The second Advanced Prostate Cancer Consensus Conference APCCC 2017 did provide a forum for discussion and debates on current treatment options for men with advanced prostate cancer. The aim of the conference is to bring the expertise of world experts to care givers around the world who see less patients with prostate cancer. The conference concluded with a discussion and voting of the expert panel on predefined consensus questions, targeting areas of primary clinical relevance. The results of these expert opinion votes are embedded in the clinical context of current treatment of men with advanced prostate cancer and provide a practical guide to clinicians to assist in the discussions with men with prostate cancer as part of a shared and multidisciplinary decision-making process.

© 2017 European Association of Urology. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

1. Introduction

The panel for the 2017 Advanced Prostate Cancer Consensus Conference (APCCC 2017) consisted of 61 multidisciplinary cancer physicians and scientists from 21 countries selected based on their academic track record and involvement in clinical or translational research in the field advanced prostate cancer (APC; Table 1).

For discussion, 10 controversial areas related to the management of men with APC that were judged to be most important for discussion were identified:

1. Management of high-risk localised and locally advanced prostate cancer
2. "Oligometastatic" prostate cancer
3. Management of castration-sensitive/naïve prostate cancer (CNCP)
4. Management of castration-resistant prostate cancer (CRPC)
5. Imaging in APC
6. Use of osteoclast-targeted therapy for skeletal related events (SRE)/symptomatic skeletal events (SSE) prevention for metastatic CRPC (mCRPC; not for osteoporosis/bone loss)
7. Molecular characterisation
8. Genetic counselling/testing
9. Side effects of systemic treatment: prevention, management, and supportive care
10. Global access to prostate cancer drugs and treatment in countries with limited resources

The consensus development process followed the procedures previously described (Supplementary data) [1]. The conference was organised around state-of-the-art lectures and presentations and debates by panellists who reviewed and discussed the evidence relevant to the above selected topics. On the last day of the conference, 150 previously agreed-upon questions were presented with options for answers in a multiple-choice format see Supplementary data. The questions were voted on publicly but anonymously.

For all questions, unless stated otherwise, responses were based on the idealised assumptions that all diagnostic procedures and treatments (including expertise in their interpretation and application) mentioned were readily available; there were no treatment contraindications and no option to include the patient in a clinical trial.

In addition, voting answers apply only to fit patients without limiting comorbidities and for patients with prostate adenocarcinoma (unless stated otherwise). When metastases were mentioned, they were detected by bone scintigraphy and/or cross-sectional imaging with computed tomography (CT) and/or magnetic resonance imaging (MRI), if not stated otherwise. Importantly, in an effort to address questions from an evidence-based and clinical utility perspective, panellists were specifically instructed not to consider cost, reimbursement, and access as factors in their deliberations, unless otherwise stated, although clearly these are critical factors in the decision making for the physician and individual patient.

Table 1 – Panel members by country and specialty

Name	First name	Speciality
Attard	Gert	Medical Oncology
Beer	Tomasz M.	Medical Oncology
Beltran	Himisha	Medical Oncology
Bossi	Alberto	Radiation Oncology, nonvoting (absence during voting)
Bristow	Rob	Radiation Oncology
Carver	Brett	Urology
Castellano	Daniel	Medical Oncology
Chung	Byung Ha	Urology
Clarke	Noel	Urology
Daugaard	Gedske	Medical Oncology
Davis	Ian D.	Medical Oncology
de Bono	Johann	Medical Oncology
Borges dos Reis	Rodolfo	Urology
Drake	Charles G.	Medical Oncology
Eeles	Ros	Clinical Oncology and Genetics
Efstathiou	Eleni	Medical Oncology
Evans	Christopher P.	Urology
Fanti	Stefano	Nuclear Medicine, nonvoting member
Feng	Felix	Radiation Oncology
Fizazi	Karim	Medical Oncology
Frydenberg	Mark	Urology
Gleave	Martin	Urology
Gillessen	Silke	Medical Oncology
Halabi	Susan	Clinical Trials and Statistics, nonvoting member
Heidenreich	Axel	Urology
Higano	Celestia S.	Medical Oncology
James	Nicolas	Clinical Oncology
Kantoff	Philip	Medical Oncology
Kellokumpu-Lehtinen	Pirkko-Liisa	Clinical Oncology
Khauli	Raja B.	Urology
Kramer	Gero	Urology
Logothetis	Chris	Medical Oncology
Maluf	Fernando	Medical Oncology
Morgans	Alicia K.	Medical Oncology and Epidemiology
Morris	Michael J.	Medical Oncology
Mottet	Nicolas	Urology
Murthy	Vedang	Radiation Oncology
Oh	William	Medical Oncology
Omlin	Aurelius	Medical Oncology, nonvoting member
Ost	Piet	Radiation Oncology
Padhani	Anwar R.	Radiology, nonvoting member
Parker	Chris	Clinical Oncology
Pritchard	Colin C.	Pathology, nonvoting member
Roach	Mack	Radiation Oncology
Rubin	Mark A.	Pathology, nonvoting member
Ryan	Charles	Medical Oncology
Saad	Fred	Urology
Sartor	Oliver	Medical Oncology
Scher	Howard	Medical Oncology
Sella	Avishay	Medical Oncology
Shore	Neal	Urology
Smith	Matthew	Medical Oncology
Soule	Howard	Prostate Cancer Foundation, nonvoting member
Sternberg	Cora N.	Medical Oncology
Suzuki	Hiroyoshi	Urology
Sweeney	Christopher	Medical Oncology
Sydes	Matthew R.	Clinical Trials and Statistics, nonvoting member
Tannock	Ian	Medical Oncology
Tombal	Bertrand	Urology
Valdagni	Riccardo	Radiation Oncology
Wiegel	Thomas	Radiation Oncology

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

The results are intended to serve only as a guide to clinicians to assist in the discussions with patients as part of a shared and multidisciplinary decision-making process. For the definitions used for APCCC 2017 please refer to Supplementary data.

The panel consisted of voting (52) and nonvoting members (9). The nonvoting members were panellists, for example, radiologists, pathologists, and statisticians who are not involved in clinical management decision making, and one clinical expert who was not present during the voting. The option "unqualified to answer" (short form "unqualified") should have been chosen if a panellist lacked experience for a specific question; the "abstain" option should have been chosen if a panellist felt unable to vote for a best choice for any reason or had prohibitory conflicts of interest. The conference also included an explicit approach to management of conflicts of interest (Supplementary data).

Detailed voting records for each of the questions brought to the panel are provided in the Supplementary data. The denominator was based on the number of panel members who voted on the particular question, excluding those who voted "unqualified to answer." In case of questions related to a topic of a previous question where only a subset of the panellists had voted for a specific answer option the votes of panel members who voted "abstain" and "unqualified to answer" were excluded.

Consensus was declared if ≥75% of the panellists who did not vote for "unqualified" or "abstain" chose the same option [2]. Throughout, the percentage of voting panellists who gave a particular response are reported, the number of voters, and the number of panellists for each answer are provided in the Supplementary data. All panellists have contributed to the designing of the questions, editing the manuscript, and have approved the final document.

Importantly, this process was uniquely able to highlight areas of disagreement and identified priorities for future clinical research, meaning areas where additional data acquisition is warranted.

2. High-risk localised and locally advanced prostate cancer

The panellists noted that there is lack of precision in the use of the term "high risk" in localised prostate cancer that is in part influenced by a discipline specific perspective. The commonly used definitions of high-risk localised patients by various societies plus the definitions used in the STAMPEDE trial are summarised in Supplementary data. High-risk localised patients have relatively good long-term outcomes [3,4]. For the APCCC 2017 conference, the European Association of Urology (EAU) guideline definition was used [5].

2.1. Pathology in locally advanced prostate cancer

Pathology reporting for radical prostatectomies (RP) should adhere to the recently published American Joint Committee on Cancer eighth edition cancer staging manual [6]. The new guidelines include the adoption of Prognostic Gleason Groups along with Gleason scores, the collapsing of pT2 to one single group, and the use of elevated prostate-specific antigen (PSA) to increase clinical staging. RP reports should comment on tumour Gleason scores using the International Society of Urological Pathology guidelines [7,8].

In men with positive lymph nodes, the total number of nodes with metastases, the tumour volume within the lymph node, and extracapsular nodal extension are poor prognostic factors [9].

In tissue from patients who have previously been treated with androgen deprivation therapy (ADT) and/or other systemic treatment or radiation therapy (RT) no Gleason score should be reported.

The panel unanimously agreed (100%) that apart from morphology and tumour stage, the following factors should be reported from a RP sample: (1) seminal vesicle involvement, (2) extraprostatic extension, (3) positive surgical margins (number, length and location, grade at margin), (4) Gleason score, and (5) grade group. There was also consensus that the following factors should be reported: (1) extent of prostatic involvement (96%), (2) number and anatomic region of resected lymph nodes and number and location of involved lymph nodes (94%), (3) tertiary Gleason grade (94%), and (4) micrometastases versus macrometastases in involved lymph nodes (81%), extranodal extension (81%), and metastatic deposits in perinodal fat tissue (79%; Table 2).

Current guidelines (EAU, National Comprehensive Cancer Network [NCCN]) recommend performing extended pelvic lymph node dissection for men with high-risk and locally APC treated by RP particularly if the risk for lymph node metastases based on available nomograms is estimated to be ≥5% despite the fact that there are no data from randomised prospective trials supporting an improvement in outcome with lymph node dissection [10–12]. The impact of minimal template versus extended lymph node dissection is not known and the pathological processing and reporting of the dissected material is not well defined.

There was a consensus (84%) that a lymph node dissection should be performed in the majority of men with cN0 cM0 high-risk prostate cancer undergoing RP whereas 9% voted for a lymph node dissection in a minority of selected patients and 5% did not vote for a lymph node dissection.

Regarding the minimum number of lymph nodes to constitute an adequate dissection in the majority of men with cN0 cM0 high-risk prostate cancer 76% of the panellists voted for a minimum of ≥11 lymph nodes (49% for 11–19 lymph nodes and 27% for ≥20 lymph nodes); 15% of the panellists voted for five to 10 lymph nodes, 9% abstained.

*Regarding the template of lymph node dissection in men with high-risk and locally advanced prostate cancer, there was a consensus that the obturator region (98%), internal iliac region (90%), and external iliac region (85%) should be dissected. Regarding the presacral lymph nodes, 51% of the panellists voted against and 46% in favour of dissection, similarly for common iliac lymph nodes 52% of the panellists voted against and 45% in favour of dissection. There was a consensus (95%) **against** routine dissection of para-aortic lymph nodes (Table 3).*

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

Table 2 – Prostatectomy pathology reporting (as clinicians, which factors do you want to be reported from a prostatectomy specimen in men with locally-advanced prostate cancer apart from morphology and tumour stage?)

Factor	Yes, useful test for majority of patients (influences your management decision; %)	Only for minority of selected patients (%)	No (%)	Abstain (%)
Seminal vesicle invasion	100	0	0	0
Extraprostatic extension	100	0	0	0
Positive surgical margins: number, length and location as well as grade at margin	100	0	0	0
Gleason score and grade group	100	0	0	0
Extent of prostatic involvement	96	2	2	0
If lymphadenectomy is performed: number and anatomic region of resected lymph nodes and number and location of involved lymph nodes	94	6	0	0
Tertiary Gleason score	94	4	2	0
In any involved lymph nodes: micro- vs macrometastases	81	9	10	0
In any involved lymph nodes: extranodal extension	81	9	10	0
In any involved lymph nodes: metastatic deposits in perinodal fat tissue	79	15	6	0
Cribriform growth pattern and intraductal tumour spread	73	14	13	0
Lymphovascular invasion	68	18	14	0
Intraductal carcinoma	67	21	12	0
Markers of inflammation (eg, inflammation within prostate cancer tissue, tumour infiltrating lymphocytes)	23	24	53	0

2.2. *Adjuvant radiation therapy after RP*

Adjuvant radiation therapy (ART) is largely considered as the administration of external beam RT in the postoperative phase in absence of objective evidence that disease has recurred or persisted. In the case of prostate cancer this would mean delivering RT when the PSA is "undetectable." Interestingly, the definition of "undetectable" has varied over the past 25 yr by nearly 100 fold from <0.3 ng/ml into the pg/ml range more recently [13].

Three randomised controlled trials have demonstrated that ART in case of unfavourable pathological features (eg, pT3b, R1) after RP delays PSA recurrence free survival; in one of these trials metastases-free survival and overall survival (OS) were also improved. Interpretation of those results is generally biased by the inclusion of men with persistent disease evidenced by low but detectable PSA levels [14–16]. Thus, in fact many of these patients treated on the ART arm should be described as receiving early salvage radiation therapy (SRT) [17,18].

Because several retrospective studies have shown that SRT, offered at PSA recurrence, may be efficient and since this approach may save some men the application of ART, many physicians defer treatment until there is evidence of recurrent disease. Unfortunately there is no prospective randomised trial comparing "pure" ART at undetectable PSA levels as currently defined versus SRT at "appropriately" low PSA levels.

Table 3 – Lymph node (LN) dissection in localised prostate cancer (which LN regions should be sampled [minimal requirement] in men with cN0 cM0 high-risk prostate cancer?)

LN region	Yes (%)	No (%)	Abstain (%)
Obturator	98	2	0
Internal iliac	90	10	0
External iliac	85	15	0
Presacral	46	51	3
Common iliac	45	52	3
Para-aortic	5	95	0

2.2.1. *ART for high risk localised prostate cancer pN0*

The topic of ART was addressed in men post-RP without lymph node involvement on surgical pathology (pN0), with undetectable postoperative PSA, and who have recovered urinary continence.

There was no consensus on ART in high-risk localised prostate cancer patients. Forty-eight percent of the panellists voted for ART for any positive surgical margins, whilst 27% of the panel voted for ART only in case of multifocal or extensive margins. Twenty-one percent of the panel did not vote for ART in this setting.

In the presence of seminal vesicle involvement alone 38% of the panel voted for ART in the majority of patients, 32% of the panel voted for ART only if combined with positive surgical margins. Twenty-six percent of the panel did not vote for ART at all in this setting.

Fifty-five percent of panellists did ***not*** *vote for ART in the case of Gleason 8–10 (Gleason Grade Group 4 or 5) as the only adverse factor, 20% of the panel voted for ART in case of Gleason 8–10 (Gleason Grade Group 4 or 5) alone for the majority of patients, and 23% in a minority of selected patients.*

Regarding radiation field, 51% of the subset of panellists who voted for ART voted for treatment of the whole pelvis and prostatic bed, while 41% voted for treating only the prostatic bed.

Thirty-six percent of the subset of panellists who voted for ART voted for adding ADT in the majority of patients, 32% in a minority of selected patients, and 32% did not vote for the addition of ADT at all. From the subset of panellists who voted for addition of ADT to ART, 69% voted for this combined treatment in men with either pT stage ≥3b and/or Gleason score ≥8 (Grade group 4–5); 28% voted for combined treatment in men with pT stage ≥3b alone independent of Gleason score;

and 3% voted for combined treatment in men with Gleason 8–10 (Gleason Grade Group 4 or 5) alone. Regarding the form of ADT 61% of the subset of panellists voted for a luteinizing hormone-releasing hormone (LHRH) agonist/antagonist, 24% for combined ADT, and 15% for an androgen receptor antagonist monotherapy. Regarding duration of ADT, 39% of the subset of panellists voted for 3–6 mo, 43% for 6–12 mo, and 18% for 18–36 mo of ADT.

2.2.2. ART for pN1 prostate cancer

For men with prostate cancer and lymph node involvement, cancer mortality rises significantly when >2 positive lymph nodes are present [19].

The question of ART in men with pN1 disease (assuming adequate lymph node sampling, section 2.1) and no local adverse factors (no pT3b, no R1) and undetectable postoperative PSA and who have recovered urinary continence was addressed by the consensus panel.

There was no consensus on ART in pN1 disease. Twenty-six percent of the panel voted for ART in men with pN1 disease in a majority of patients, 29% voted for ART in a minority of selected patients, while 43% of the panel did not vote for ART in this setting.

Regarding radiation field, 97% of the subset of panellists who voted for ART voted for the whole pelvis plus prostatic bed as radiation field.

The subset of panellists who voted for ART also voted on factors that influenced their decision to recommend ART: 62% voted for taking both the number and location of positive lymph nodes into consideration when recommending ART, 33% based their decision only on the number of involved lymph nodes, and 5% only on the location of involved lymph nodes. Fifty percent of this subset of panellists voted for ART in men with one or two positive lymph nodes in the presence of intermediate- or high-grade, nonorgan-confined disease and in those with three to four lymph nodes irrespective of grade and T-stage, 17% voted for ART in all patients, 15% voted for ART in patients with ≤2 positive lymph nodes independent of grade and T-stage, and 15% in patients with ≤4 positive lymph nodes independent of grade and T-stage.

Of the panellists who voted for ART for pN1 disease, 100% voted for adding ADT to ART. Regarding the duration of ADT in this situation, 18–36 mo was voted for by 57% of these panellists, 6–12 mo by 30%; 11% voted for 3–6 mo, while 2% voted for life-long ADT.

2.3. Salvage radiation therapy after RP

While RP generally yields excellent results in patients with localised prostate cancer, the recurrence rates after RP for high-risk prostate cancer may rise as high as 50–80% [15]. In the case of recurrence, SRT is a treatment option [20].

The appropriate PSA level at which to initiate SRT is still unclear. European guidelines recommend initiating SRT before the post-RP PSA level exceeds 0.5 ng/ml, whilst NCCN guidelines recommend SRT in patients with confirmed increasing PSA [21,22].

Two multi-institutional retrospective studies showed an improved freedom from biochemical progression and distant metastases following very early SRT at a PSA <0.2 ng/ml as opposed to patients in which SRT was initiated at a PSA level of 0.2–0.5 ng/ml versus higher PSA values [23,24]. Such analyses are confounded by lead-time and length-time bias and the topic remains an area of uncertainty.

According to the current EAU guidelines, the SRT dose should be at least 66 Gy but the optimal dose may be higher; the optimal dose and fractionation is unclear and is being addressed in several ongoing trials.

Combining SRT with ADT may be an option, particularly in men with high-risk disease. In the GETUG-AFU 16 trial, the 5-yr freedom from biochemical progression was 80% with SRT plus 6 mo of ADT versus 62% with SRT alone [25]. In the RTOG 9601 trial, OS was improved with SRT plus 2 yr of high-dose bicalutamide (150 mg daily) compared with SRT plus placebo but a significant proportion of included men had PSA levels ≥0.7 ng/ml [26].

Regarding the confirmed PSA level at which to initiate SRT, 44% of the panel voted for 0.2 ng/ml, whilst 38% voted for 0.1 ng/ml, 10% voted for 0.5 ng/ml, and 4% for <0.1 ng/ml.

The panel reached no consensus regarding a level of PSA above which SRT would not be recommended. Twenty-five percent of the panellists considered 2 ng/ml the maximum value, 19% considered 1 ng/ml the maximum value, 11% chose 0.5 ng/ml as a maximum value, and 19% of the panel voted that there should be no maximal upper limit of PSA.

The subset of panellists who voted for SRT also voted on the addition of ADT. Sixty-one percent voted for ADT in the majority of men, 29% in a minority of selected patients, for example, based on PSA level and PSA doubling-time, and 10% of these panellists did not vote for the addition of ADT. Regarding the duration of ADT in combination with SRT, 34% of these panellists who opted for the addition of ADT voted for 3–6 mo, 41% for 6–12 mo, and 25% for 18–36 mo of ADT.

2.4. Discussion of high-risk localised and locally advanced prostate cancer

The consensus questions focused on men undergoing RP and the topics of ART and SRT. The choice of primary treatment of high-risk and locally advanced prostate cancer is also an area of controversy, but was not addressed at this conference.

The votes of the panel showed a consensus on the required information for pathology reporting in men undergoing a RP.

There was a lack of consensus regarding the role of ART and SRT reflecting the many uncertainties and multiple unanswered questions in both topics. One of the reasons for uncertainty is that the ART trials did not have an early SRT arm as a comparator and as such are not comparable to current practice. Another weakness of these trials is the relatively high PSA at which "adjuvant" RT was started, again not comparable to current practice.

As with any adjuvant treatment, ART bears the risk of overtreatment and can result in acute side effects as well as deleterious effects on long-term functional outcome

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

(eg, potency, continence) but such potential risks must be balanced against the potential benefits, namely improved oncological outcomes [18,27,28].

The quest to define "unnecessary" RT and how to select which patients really require ART and for which patients SRT is appropriate is currently ongoing. Several well-powered phase 3 trials (RADICALS, RAVES, and GETUG-17) will provide evidence on which to base updated discussions.

In the meantime, regarding SRT, recent retrospective studies suggest that initiating SRT at lower PSA values (< 0.2 ng/ml) improves biochemical progression free survival as compared with using the traditional recommended confirmed value of 0.2 ng/ml and rising for definition of biochemical relapse (BCR) [23,24]. These data were reflected by the votes of the panel wherein a significant proportion of panellists would initiate SRT below the PSA threshold recommended by current guidelines.

The addition of ADT to RT as primary treatment of the prostate is a well-established concept [29–33]. But the addition, timing, and duration of ADT, specifically for ART but also for SRT, are less well examined [26]. Accordingly, there was no consensus regarding the role of adding ADT to ART and SRT.

Prospectively validated prognostic and predictive molecular biomarkers are required that will improve the performance of clinical and pathological features but this can only be determined in the context of large phase 3 randomised trials with adequate long-term follow-up. Additionally, the increasing use of next-generation imaging methods in combination with more sensitive PSA assays may also alter treatment approaches in the future.

3. Oligometastatic prostate cancer

3.1. Definition of oligometastatic prostate cancer

Hellman and Weichselbaum [34] proposed the term "oligometastases" in 1995 for defining a disease stage with a limited number of clinically detectable metastases.

The biological definition of oligometastatic prostate cancer is open to interpretation as is the entire concept that this is a prognostic and therapeutically distinct subset of patients that falls somewhere in-between localised and metastatic disease. No formal cut-off for "oligo" has been defined in the literature [35]. Some definitions incorporate both the site of metastases in addition to the number of lesions to define the oligometastatic state [35,36]. Variables to include in the description of men with oligometastatic disease include: the distinction of synchronous versus metachronous metastases, the number and site of lesions, and whether the patient is castration-naïve or castration-resistant [36]. Of importance is also the imaging method used to define oligometastatic disease. Newer imaging techniques will detect more metastases in many patients classified as "oligometastatic" by conventional imaging (CT and bone scintigraphy). Many patients considered as M0 on conventional imaging may turn out to have oligometastatic disease especially when imaging is performed at lower PSA levels than in the past.

The panel did not reach consensus on what constituted the definition of oligometastatic disease. Sixty-one percent of the panellists voted for a limited number of bone and/or lymph nodes as a clinically meaningful definition of oligometastatic prostate cancer that influences treatment decisions (local ablative treatment of all lesions ± systemic therapy), 10% of the panellists voted for an oligometastatic definition which includes only patients with a limited number of lymph node metastases, 13% voted for patients with a limited number of metastases at any location (including visceral disease), and 10% of the panellists did not believe that oligometastatic prostate cancer exists as a clinically meaningful entity.

The subset of panellists who believed in the concept of oligometastatic prostate cancer voted on the number of lesions. Regarding the cut-off for the number of metastases to consider a prostate cancer patient as oligometastatic 14% voted for ≤2 metastases, 66% for ≤3 metastases, and 20% of these panellists voted for ≤5 metastases as a cut-off. Of the panellists believing in the oligometastatic concept, 52% voted for a biopsy (if feasible) of an oligometastatic lesion for diagnostic purposes in a minority of selected patients, while 34% voted for biopsy in the majority of patients and 14% of these panellists did not vote for a biopsy.

3.2. Synchronous "oligometastatic" castration-naive prostate cancer

This section addresses patients diagnosed with de novo apparent oligometastatic disease in the castration-naïve state, that is, they present with synchronous oligometastases and an untreated primary. In such patients, no prospective randomised data are available to show a benefit for ablative treatment of all lesions including the primary—either with or without systemic therapy.

For men who present with de novo oligometastatic disease, a total of 25% of the panellists voted for lifelong ADT ± six cycles of docetaxel without local ablative treatment. Eight percent of panellists voted for local ablative treatment of all lesions including the primary (surgery or RT) without any systemic treatment, 22% of panellists voted for local ablative treatment with a short course (6–12 mo) of ADT ± docetaxel, 31% of panellists voted for local ablative treatment and an intermediate long course (24–36 mo) of ADT ± docetaxel, 8% of panellists voted for local ablative treatment and life-long ADT ± docetaxel.

Among the panellists who voted for local ablative treatment plus ADT in men with de-novo oligometastatic prostate cancer and an untreated primary, 28% voted for the addition of docetaxel in the majority of patients, 39% voted for the addition of docetaxel in a minority of selected patients; 33% of these panellists did not vote for the addition of docetaxel in this situation. If they voted for treatment of the primary tumour in this situation, 45% voted for RT, 22% voted for surgery, and 31% voted for either RT or surgery.

3.3. Metachronous oligometastatic castration-naïve prostate cancer

This section addresses men who present with recurrent apparent oligometastatic prostate cancer in the castration-

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

naïve state; that is, they present with metachronous metastases after local treatment of the primary. No prospective randomised data are available to show a benefit for radical ablative treatment of all lesions with or without systemic therapy as compared with standard of care (ADT ± docetaxel) [37]. A meta-analysis of 20 small studies of local lymph node only recurrence after primary treatment suggested that, despite a lack of high-level evidence, ablative node-directed therapy may yield in good short-term oncologic outcomes and may defer the need for systemic treatment [38].

There was no consensus on treatment options. For treatment of men with asymptomatic oligometastatic recurrent CNPC 32% of the panel voted for systemic therapy with lifelong ADT ± docetaxel without local ablative therapy of the metastases. Twelve percent voted for local ablative therapy of the metastases without additional systemic therapy, while 30% voted for local ablative therapy with a short course (6–12 mo) of ADT ± docetaxel, 18% for local ablative therapy with a longer course (24–36 mo) of ADT ± docetaxel, and 4% voted for local ablative therapy and lifelong ADT ± docetaxel.

Among the panellists who voted for local ablative treatment in men with oligometastatic recurrent CNPC limited to lymph node metastases in the pelvis, 23% voted for salvage lymph node dissection, 19% for salvage lymph node dissection plus RT to the pelvis (if no prior whole-pelvis RT), 16% of these panellists voted for focal RT, and 42% for whole pelvis RT (if no prior whole-pelvis RT) ± a boost to the suspicious nodes.

3.4. Rising PSA on ADT (mCRPC) and oligometastatic disease

This section addresses patients diagnosed with oligometastatic disease progression in the castration resistant state. No prospective randomised data are available demonstrating a benefit for local radical treatment of all lesions in addition to ADT, compared with standard of care, that is, the addition of a new systemic treatment to ADT.

Among the panellists who believed that oligometastatic mCRPC is a meaningful entity there was no consensus on treatment options. Forty-four percent of these panellists voted for continuation of ADT and adding additional systemic therapy, 29% for local ablative treatment of all lesions in combination with ongoing ADT and addition of systemic treatment, 25% for local ablative treatment of all lesions while continuing ADT without addition of systemic treatment and 2% voted for local ablative treatment of all lesions and the cessation of ADT.

3.5. Discussion of oligometastatic prostate cancer

In addition to prostate cancer, the oligometastatic state is of interest in a growing number of other cancer types, for example, breast, renal cell, colorectal, gastric, and non-small cell lung cancer. Like in prostate cancer, in these diseases the majority of data are retrospective in nature and therefore difficult to interpret. In some cases, treatment of local disease appears to be associated with long-term survival. Prospective trials are ongoing in several of these entities.

The concept of oligometastases implies that a local therapy directed at the primary cancer and/or metastases might improve survival though there is no strong evidence to support this. There was no consensus on treatment options, but from the voting it seems that the enthusiasm for the topic exceeds the evidence reported to date. The available data are not prospective, are subject to selection bias, and thus require validation in prospective randomised controlled trials. Such trials should focus on OS as an endpoint, since earlier endpoints such as progression-free survival (PFS) or time to systemic therapy are not well defined and their clinical importance is less clear. Distinguishing between synchronous and metachronous lesions, and separating pelvic nodal relapse from M1 disease is also likely to be important. Studies of patients with oligometastatic disease are of increasing importance, since more sensitive imaging techniques are anticipated to increase the proportion of men with radiographically detected lesions. At the very least, until randomised clinical trial data are available, large collaborative national and international registries of men treated for oligometastatic prostate cancer should be initiated to prospectively collect data on consecutively treated patients.

4. Castration-naive prostate cancer

There was inconsistent use in discussions of the terms castration-naïve or castration-sensitive, to designate prostate cancer either not previously treated with ADT, or cancers demonstrating ongoing sensitivity to ADT. The term castration-naïve is used in this manuscript for simplicity to cover both clinical scenarios.

4.1. When to start ADT (post-RP ± RT or post RT)

The optimal timing of initiation of ADT, duration, specific ADT modality, and the indications for initiating ADT are not well defined. For patients presenting with metastases with impending complications and especially if symptomatic, an initial short course of AR antagonist treatment to prevent the unwanted clinical consequences of testosterone surge is recommended when LHRH agonists are initiated.

For patients with BCR, the decision to initiate ADT will likely depend upon several parameters including life expectancy, time to PSA relapse after local therapy, PSA kinetics, absolute PSA level, age, sexual function, baseline fatigue, cardiovascular risk, and neurologic and cognitive status. For patients with BCR without overt metastatic disease, the decision to proceed with intermittent ADT versus continuous ADT should also be considered.

In men with nonmetastatic disease and confirmed rising PSA (postlocal therapy ± SRT), 65% of the panellists voted for the initiation of ADT only in a minority of selected men, for example, in case of a PSA ≥4 ng/ml and rising with doubling time less than 6 mo or a PSA ≥20 ng/ml (STAMPEDE inclusion criteria). Twenty-one percent voted for starting ADT in the majority of men irrespective of these factors and 12% voted for starting ADT only after detection of metastases.

4.1.1. Monitoring of testosterone

Current data do not provide clarity regarding the optimal level of testosterone suppression to be achieved in men

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

with advanced prostate cancer on ADT. The regulatory-approved level of less than 50 ng/dl, per Food and Drug Administration and European Medicines Agency, was based upon the initial leuprolide registration trial and 50 ng/dl was the lowest limit of detection of the radioimmunoassay used at that time [39]. Ensuing trials have suggested that reaching a testosterone level of ≤20 ng/dl may achieve a delay in time toward the development of castration resistance; however, this threshold, as well as the interval at which to measure serum testosterone levels remains uncertain [40].

In men with prostate cancer responding to ADT, 44% of the panel voted for regular monitoring of testosterone levels (apart from measuring testosterone at biochemical progression) and 34% of the panellists voted for measuring testosterone in a minority of selected patients (eg, failure to achieve PSA nadir < 0.2 ng/ml), 22% of the panel did not vote for regular testosterone measurement in responding patients.

Fifty-four percent of the panel voted for a testosterone level <50 ng/dl (< 1.73 nmol/l) as appropriate for men on ADT, 36% voted for a testosterone level <20 ng/dl (< 0.69 nmol/l), while 10% abstained.

There was no consensus on the therapeutic approach to men with rising PSA on a LHRH agonist whose testosterone level is confirmed as being noncastrate (apart from ruling out application errors and/or poor compliance). Despite the lack of evidence, 36% of the panel voted for a change to a LHRH antagonist, 26% for addition of a first-generation AR antagonist, 20% for a change to an alternative LHRH agonist, and 14% voted for orchiectomy.

4.2. Chemotherapy in castration-naïve nonmetastatic prostate cancer

There is some evidence to support combination treatment as an upfront alternative to single-modality therapy for men who present with high-risk localised prostate cancer. Such approaches generally combine ADT with RT and docetaxel-based chemotherapy. A total of three randomised trials in such patients have been reported. The GETUG-12 trial showed an improvement in failure-free survival (FFS) with four cycles of docetaxel and estramustine plus ADT as compared with ADT alone [41,42]. The second trial, RTOG 0521, so far only presented as an abstract, examined the combination of six cycles of adjuvant docetaxel postradical RT with ADT for 24 mo (NCT00288080). The STAMPEDE trial allowed inclusion of high-risk localised as well as biochemical recurrent and metastatic patients. The number of events for definitive interpretation of survival of M0 patients in the docetaxel arm of STAMPEDE is too low and no conclusions regarding the effect of addition of docetaxel on OS in this trial can be drawn [43].

A meta-analysis reported a consistent effect on FFS for chemo-hormonal therapy in the M0 subgroup as opposed to ADT alone [44]. Data for OS are not yet mature.

For men with N1 M0 CNPC, 71% of the panel did not vote for the addition of docetaxel to ADT, 25% voted for the addition in a minority of minority of selected patients, and 4% for the majority of patients.

For men with biochemical relapse only, there was a consensus (90%) for not adding docetaxel to ADT.

4.3. Castration-naive prostate cancer M1 (metastatic)

Testosterone suppression alone has long been the standard treatment for patients with metastatic prostate cancer commencing systemic treatment [45]. Although the majority of men with mCNPC experience a PSA decline with ADT, the median FFS in a cohort of newly diagnosed mCNPC was approximately approximately 1 yr, with a wide range [46]. Subgroup analyses from recent clinical trials showed that higher volume of metastases and presentation with de novo metastatic disease are risk factors associated with a shorter OS with ADT alone. Other purported poor prognostic clinical factors include higher Gleason score, pain, and elevated alkaline phosphatase [45,47,48].

Docetaxel given at the start of ADT was the first drug shown to improve the OS of men with mCNPC in two large trials [43,49]. The first phase 3 study of docetaxel in mCNPC, GETUG 15, showed an improvement in PFS but not OS [47].

There is ongoing discussion on the definition of "high-volume" disease and whether there is a definition that is prognostically relevant or predictive of treatment benefit.

For a definition of high-volume disease, 74% of the panellists voted for the definition, as used in CHAARTED (visceral [lung or liver] and/or ≥ 4 bone metastases, at least one beyond pelvis and vertebral column), either with standard imaging (59%) or with any imaging (15%), 6% voted for the high-volume definition developed by SWOG (visceral [lung or liver] and/or any appendicular skeletal involvement) and 6% voted for a simplified version of high-volume of visceral and/or ≥4 bone lesions regardless of distribution and imaging used. Fourteen percent of the panellists had the opinion that high-volume disease is not a clinically meaningful entity.

For men with high-volume mCNPC, 68% of the panellists voted for continuous ADT using a LHRH agonist (plus a short course of first-generation AR antagonist to prevent testosterone surge) as their preferred hormone therapy, another 10% for starting with an LHRH antagonist (no flare-up prevention needed) and switching to an LHRH agonist in the course of treatment. Continuous LHRH antagonist treatment was voted for by 6%, orchiectomy by 2%, and continuous combined ADT by 14% of the panellists. None of the panellists voted for any form of intermittent ADT or AR-antagonist monotherapy in the high-volume M1 setting.

Not all men are suitable for chemotherapy with docetaxel and the criteria rendering a patient "unsuitable" for docetaxel are not well defined.

The panel voted on factors they would consider rendering a man "unfit" for docetaxel.

There was a consensus for severe hepatic impairment (96%), neuropathy grade ≥2 (82%), and platelets $<50 \times 10^9$/l and/or neutrophils $<1.0 \times 10^9$/l (81%). For the other proposed factors alone there was no consensus (Table 4).

In the original publication of the CHAARTED trial, the subgroup of men with high-volume disease showed a clinically significant survival benefit and the point estimate for the low volume patients was the same in that

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

Table 4 – Definition "unfit" for docetaxel

What are meaningful definitions "not being suitable for docetaxel", apart from allergy to the substance ("docetaxel ineligible")?	Yes (%)	Only in combination with other factors (%)	No (%)	Abstain (%)
Severe hepatic impairment (eg, ALT/AST > 5 × ULN and/or bilirubin > 3 × ULN)	96	2	2	0
Neuropathy grade ≥2	82	18	0	0
Platelets $<50 \times 10^9$/l and/or neutrophils $<1.0 \times 10^9$/l	81	15	4	0
Frailty assessed by geriatric or other health status evaluation	69	29	2	0
Performance status ≥2 for reasons other than cancer	62	32	4	2
Moderate hepatic impairment (eg, ALT/AST > 3–5 × ULN and/or bilirubin > 1.5–3 × ULN)	52	48	0	0

ALT = alanine transaminase; AST = aspartate transaminase; ULN = upper limit of normal.

publication, albeit with much wider confidence intervals [49]. No OS benefit has yet been demonstrated for early docetaxel use with longer-term follow-up in subgroup analyses performed in both the low-volume mCNPC cohorts of the GETUG 15 (posthoc) or CHAARTED (prespecified) trials [49,50]. Further subgroup analyses for men with de novo metastatic prostate cancer (majority of included patients) versus men with relapse after local treatment were presented but have not yet been published (European Society for Medical Oncology 2016 and GU-ASCO 2017).

The large STAMPEDE trial included both M0 and M1 patients and no heterogeneity of treatment effect was observed.

For men who are suitable for chemotherapy and have de novo mCNPC and high-volume disease as defined by CHAARTED, there was a consensus (96%) for addition of docetaxel to ADT in the majority of patients, 4% voted for docetaxel in a minority of these men. For the other subgroups of mCNPC there was no consensus (Table 5). There was consensus that men with biochemical relapse (N0M0) should not receive docetaxel in addition to ADT.

If chemo-hormonal treatment is used in men with mCNPC there was consensus (78%) that docetaxel should be started within 3 mo of starting ADT and 20% of these panellists voted for starting even within 2–4 wk. Within 4 mo was considered sufficient for another 18% of the panellists.

In the subset of panellists who voted for chemo-hormonal therapy there was also consensus (96% of the panel) for the 3-weekly regimen of docetaxel with 75 mg/m^2. Only 4% of the panel voted for the use of the 2-weekly regimen with 50 mg/m^2.

Docetaxel in the 3-weekly regimen does not bear a high risk (>20%) of febrile neutropenia; however, according to existing guidelines (NCCN, ESMO, ASCO), primary granulocyte-colony stimulating factor prophylaxis should be considered in men with risk factors namely prior chemo- or RT, bone marrow involvement by tumour, renal dysfunction (creatinine clearance < 50 ml/min), or age >65 yr, and receiving full chemotherapy dose and intensity. It is not uncommon that such risk factors are present in men with APC. Of note, there is preclinical data suggesting that myeloid-derived suppressor cells, which may play a role in

Table 5 – Chemo-hormonal therapy with docetaxel

Do you recommend docetaxel in addition to ADT:	Yes, in the majority of patients (%)	In a minority of selected patients (%)	No (%)	Abstain (%)	Unqualified to answer (%)
In men with de novo metastatic castration-naive prostate cancer and high-volume disease as defined by CHAARTED (visceral metastases and/or ≥ 4 bone lesions with ≥ 1 beyond vertebral bodies and pelvis)?	96	4	0	0	0
In men with de novo metastatic castration-naive and low-volume disease as per CHAARTED?	29	65	9	0	0
In men with metastatic castration-sensitive/naive disease relapsing after prior treatment for localised prostate cancer and with high-volume disease as per CHAARTED?	74	24	2	0	0
In men with metastatic castration-sensitive/naive disease relapsing after prior treatment for localised prostate cancer with low-volume bone metastases as per CHAARTED criteria?	19	54	25	2	0
In men with castration-sensitive/naive N1 M0 prostate cancer?	4	25	71	0	0
In men with castration-sensitive/naive N0 M0 (nonmetastatic) prostate cancer with biochemical relapse?	0	10	90	0	0

ADT = androgen deprivation therapy.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

cancer progression can be influenced by granulocyte-colony stimulating factor [51–53].

In the subset of panellists who voted for chemo-hormonal therapy, 6% voted for white blood cell (WBC) growth factors from start of therapy in the majority of patients, 50% for a minority of selected patients. Forty-four percent of these panellists did not vote for WBC growth factors from start of therapy.

Regarding concomitant steroid dosing the CHAARTED and GETUG-15 trials did not require daily steroids, whereas STAMPEDE required prednisone (10 mg) daily.

In the subset of panellists who voted for chemo-hormonal therapy, 58% voted for prescribing the 3-weekly docetaxel regimen with no daily steroid in the chemo-hormonal setting and 38% with 10 mg prednisone daily.

4.4. Local therapy in men with mCNPC

The current standard of care for patients presenting with de novo metastatic prostate cancer is ADT with or without docetaxel (section 4.3). Transurethral resection of the prostate may be used to palliate local symptoms. The rationale for potentially using a local ablative treatment (external beam RT or RP) in these patients is based on several considerations. Significant morbidity related to local symptoms including pain, obstructive urinary symptoms, and haematuria can occur in these men, either when the cancer is diagnosed or when it progresses later in the disease course [54]. A local ablative treatment used upfront may prevent these adverse events, as suggested in a retrospective analysis [55]. Local treatment, however, can add considerable toxicity.

In men with mCNPC, there is no randomised prospective data to support local ablative treatment of the primary. Retrospective studies based on registries, while biased by design, suggest a survival benefit when a local treatment is applied upfront [56–58]. Similar findings were reported in men with nodal disease treated locally with either RT or RP [38,59,60]. These results have to be interpreted with caution and treatment of the primary for this specific disease state should only be done in the context of a clinical trial.

*Fifty-two percent of the panel was **against** treating the primary tumour in addition to systemic therapy in men with de novo high-volume mCNPC who are not symptomatic from their primary, 38% voted for treating the primary in a minority of patients, 10% in the majority of patients in this situation.*

In the subset of panellists who voted for treatment of the primary in this situation, 71% voted for RT, 26% voted for a RP; 3% voted for other treatments.

4.5. Discussion of CNPC

In summary, although docetaxel-based CNPC studies have provided evidence that some patients benefit from early docetaxel, the field is rapidly evolving and a number of unanswered questions have emerged [44]. Less than a third of the panel recommended addition of docetaxel to ADT in the majority of patients with low-volume metastatic disease despite the fact that use of data from subgroups has limitations and is considered hypothesis generating. Importantly, there is probably significant overlap between patients called "low-volume" metastatic and "oligometastatic." The panel seemed more conservative in relation to addition of docetaxel than in relation to local ablative treatment.

Additional studies are needed to focus on identifying more accurate biomarkers and better understanding of the underlying mechanisms of resistance to ADT to define a more precise therapeutic strategy for a given biological driver for a given cancer and therefore the biological basis for the benefit of AR targeting and cytotoxic treatment of their prostate cancer [61–63]. Moreover, since the studies of ADT plus abiraterone in the mCNPC setting have shown an overall survival benefit, further work will be required to determine the role of docetaxel either with ADT alone or with ADT plus abiraterone [64,65]. Further studies with other AR targeted agents including the combination with chemotherapy are ongoing in the same setting.

Despite the lack of prospective data from randomised trials, a rather high percentage of the panel would consider treatment of the primary tumour in some men with metastatic disease. Applying such a local ablative treatment to men with metastases in a nonresearch setting could be "excessive" in terms of treatment burden and is unproven but some panellists have voted for this approach, not only in the oligometastatic setting, but also in the general metastatic setting, and this seems to be done in clinical practice all over the world.

This "try it because you believe it" approach is well-intentioned but may result in adverse consequences for patients, in some cases on a large scale, as in the gross overtreatment of low-risk localised prostate cancer. In the era of evidence-based medicine, this approach is disappointing and we, as a scientific community, should do everything we can to avoid having this happen again. It is worth remembering that in other malignancies, for example, in metastatic breast cancer, retrospective data and even a meta-analysis had similarly suggested an OS benefit with locoregional treatment in metastatic disease that was not confirmed in a randomised prospective study [66]. Despite a large percentage of the panel considering treatment of the primary in the metastatic setting, there is still an overwhelming recommendation that this question for prostate cancer has to be answered in prospective randomized trials before being widely adopted in clinical practice. Several such trials are currently testing whether a local definitive treatment directed to the prostate primary cancer can improve patient outcome in men with mCNPC (eg, NCT00268476, NCT01957436, NCT02454543; ISRCTN06890529).

5. Castration-resistant prostate cancer

5.1. Sequencing and combinations in mCRPC

The field of prostate cancer drug development has seen remarkable progress in the past 10 yr. However, this

progress is largely based on registration studies conducted by a "one size fits all" approach in a regulatory framework that focused on prior therapy with ADT and docetaxel exposure rather than one defined by individual patient biology. With current knowledge about heterogeneity in prostate cancer, future registration trials will need to have more specific eligibility criteria related to the mechanism of action of the drug being studied.

Because the registration trials for each of these agents were conducted contemporaneously, the question of sequencing of the available treatment options is still relevant. The earlier inclusion of docetaxel as part of a chemo-hormonal therapy regimen in CNPC (section 4.2 and 4.3) may have implications on subsequent treatment choices. None of the registration trials for agents in the CRPC setting included such patients.

5.1.1. First-line treatment for men with mCRPC

Several prospective randomised phase 3 trials showed an OS benefit for first-line treatment in men with mCRPC. None of the control arms used in these trials is currently considered standard of care. Abiraterone, enzalutamide, and sipuleucel-T were evaluated as first-line agents in asymptomatic patients, docetaxel in both symptomatic and asymptomatic patients, and radium-223 dichloride (radium-223) in symptomatic patients with bone metastases [67–72]. Sipuleucel-T is only available in the USA.

Another first-line trial testing cabazitaxel in two different doses versus docetaxel has been reported and failed to show superiority of cabazitaxel, but has not yet been published (NCT01308567, ASCO 2016).

There was consensus that asymptomatic men with mCRPC should receive abiraterone or enzalutamide as first-line treatment. This recommendation was independent of whether they had received ADT alone (86%) or ADT plus docetaxel (90%) in the castration-naïve setting.

In case of progression within 6 mo after completion of docetaxel in the castration-naïve setting in an asymptomatic man, 77% of the panellists voted for abiraterone or enzalutamide as first-line mCRPC treatment, 17% voted for cabazitaxel, and 2% each docetaxel or platinum-based chemotherapy (Table 6).

5.1.2. Second-line treatment for men with mCRPC

There are only prospective randomised data for second-line treatment in men who have received docetaxel as first-line treatment for mCRPC. In this setting, abiraterone, cabazitaxel, enzalutamide, and radium-223 (about half of the patients included were pretreated with docetaxel) have shown an OS benefit [72–75]. Currently, most patients are treated with abiraterone or enzalutamide in the first-line setting and there is not a lot of prospective data on second- or further-line treatment in these men.

In symptomatic men who had primary resistance to first-line treatment with abiraterone or enzalutamide there was a consensus (96% of the panel) for treatment with a taxane.

In symptomatic men who had acquired resistance to first-line abiraterone or enzalutamide there was a consensus (90% of the panellists) for a taxane, 8% voted for radium-223, and 2% had no preferred option.

Table 6 – Sequencing of metastatic castration-resistant prostate cancer (mCRPC) first-line options

What is your preferred first-line mCRPC treatment option:	Abiraterone or enzalutamide (%)	Cabazitaxel (%)	Docetaxel (%)	Platinum-based chemotherapy (%)	Radium-223 (%)	Sipuleucel-T (%)	No preferred option (%)	Abstain (%)	Unqualified to answer (%)
In the majority of asymptomatic men who did not receive docetaxel in the castration-naive setting?	86	0	6	0	0	8	0	0	0
In the majority of symptomatic men who did not receive docetaxel in the castration-naive setting?	52	0	46	0	0	0	2	0	0
In the majority of asymptomatic men who did receive docetaxel in the castration-naive setting?	90	2	2	0	0	6	0	0	0
In the majority of symptomatic men who did receive docetaxel in the castration-naive setting?	73	19	6	0	2	0	0	0	0
In the majority of asymptomatic men who received chemo-hormonal therapy and who progressed within ≤6 mo after completion of docetaxel in the castration-naive setting?	77	17	2	2	0	0	2	0	0
In the majority of symptomatic men who received chemo-hormonal therapy and who progressed within ≤6 mo after completion of docetaxel in the castration-naive setting?	57	27	0	4	8	2	2	0	0

In asymptomatic men with disease progression on or after first-line docetaxel for mCRPC, there was a consensus (92%) for abiraterone or enzalutamide as second-line treatment. Only 6% of the panellists voted for treatment with cabazitaxel and 2% for radium-223.

In symptomatic men with disease progression on or after first-line docetaxel for mCRPC there was consensus (76%) for treatment with abiraterone or enzalutamide, 18% voted for cabazitaxel and 6% voted for radium-223 (Table 7).

5.1.3. Third-line treatment for men with mCRPC

There are no randomised prospective data for third-line treatment in mCRPC.

In a man who has received abiraterone or enzalutamide as first-line treatment, and docetaxel as second-line treatment, 61% of the panellists voted for treatment with cabazitaxel, 15% for radium-223, 8% voted for abiraterone or enzalutamide (depending on which has already been used), 8% had no preferred choice, and 6% voted for a platinum-based chemotherapy.

Platinum compounds have been studied in a variety of monotherapy schedules and in different combinations and clinical disease stages in men with APC [76]. In unselected patients the response rates to platinum compounds are not convincing and derived from mostly small clinical trials.

In men with mCRPC who have exhausted approved treatments and if no clinical trial was available a total of 96% of the panellists voted for a carboplatin-based chemotherapy in certain situations: 33% in the majority of patients, 2% only in patients with DNA repair defects, 14% only in patients with neuroendocrine differentiation or clinical evidence suggestive of neuroendocrine differentiation (eg, atypical pattern/distribution of metastases, rapid progression without correlation with PSA kinetics; sudden onset of rapid growth of visceral metastases or multiple lytic bone metastases; presence of paraneoplastic syndromes), and 47% in patients with DNA repair defects and/or neuroendocrine differentiation or suggestion thereof.

5.2. Treatments and schedules for mCRPC

Newer androgen-receptor pathway targeted therapies such as enzalutamide or abiraterone carry risks of class-specific adverse events. Abiraterone adverse events include those related to mineralocorticoid excess, hypertension, cardiac and liver dysfunction, and fluid retention. Enzalutamide can be associated with fatigue, hypertension, cognitive and mood impairment, falls, and fractures. Both drugs carry the risk of pharmacokinetic drug-drug interactions that can increase the risk of toxicity particularly in older men treated with multiple other drugs.

Abiraterone and enzalutamide have been developed almost simultaneously and there are no published randomised prospective trials available that compare these two agents against each other.

Asked about their preferred choice between abiraterone and enzalutamide for first-line treatment of men with mCRPC and no contraindication to either drug, 35% of the panellists voted

Table 7 – Sequencing of metastatic castration-resistant prostate cancer (mCRPC) second-line options

What is your preferred second-line mCRPC treatment option:	Abiraterone or enzalutamide (depending which has already been used; %)	Taxane (%)	Radium-223 (%)	Sipuleucel-T (%)	No preferred option (%)	Abstain (including other treatment option) (%)	Unqualified to answer (%)
In the majority of men with asymptomatic mCRPC who had progressive disease as best response to first-line abiraterone or enzalutamide?	14	70	4	6	6	0	0
In the majority of men with symptomatic mCRPC who had progressive disease as best response to first-line abiraterone or enzalutamide?	0	96	4	0	0	0	0
In the majority of men with asymptomatic mCRPC and secondary (acquired) resistance (initial response followed by progression) after use of first-line abiraterone or enzalutamide?	27	57	10	4	2	0	0
In the majority of men with symptomatic mCRPC and secondary (acquired) resistance (initial response followed by progression) after use of first-line abiraterone or enzalutamide?	0	90	8	0	2	0	0
In the majority of asymptomatic men, progressing on or after docetaxel for mCRPC (without prior abiraterone or enzalutamide)?	92	6	2	0	0	0	0
In the majority of symptomatic men with mCRPC, progressing on or after docetaxel for mCRPC (without prior abiraterone or enzalutamide)?	76	18	6	0	0	0	0

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

for abiraterone, 24% for enzalutamide, and 37% had no preferred choice.

The panellists were also asked to vote for their preferred choice between abiraterone and enzalutamide in patients with special situations (mainly comorbidities; Table 8).

There was a consensus for abiraterone over enzalutamide in men with a history of falls (94%), significant baseline fatigue (88%), and significant neurocognitive impairment (84%). There was consensus for enzalutamide over abiraterone in men with diabetes mellitus requiring prescription drug therapy (84%; Table 8).

The preferred glucocorticoid regimen when starting abiraterone was prednisone 10 mg daily for 67% of panellists and 5 mg daily for 27% of the panellists. Six percent voted for dexamethasone.

There is a retrospective analysis of patients on abiraterone plus prednisone who had PSA progression with or without progression by imaging but in the absence of clinical progression. In these patients abiraterone was continued and prednisone was switched to 0.5–1 mg dexamethasone/d. There were responses demonstrated by PSA as well as by imaging [77,78]. The level of evidence for this intervention is low.

In men with mCRPC who are asymptomatic and have a rising PSA on abiraterone plus prednisone, 37% of the panellists voted for a steroid switch to dexamethasone in the majority of patients, 35% in a minority of selected patients, and 26% did not vote for a steroid switch.

The pivotal trial which led to the registration and approval of docetaxel in men with mCRPC included two regimens: docetaxel 75 mg/m^2 every 3 wk and a weekly docetaxel schedule of 30 mg/m^2 (d 1, d 8, d 15, d 22, and d 29 of a 6-wk cycle) both with prednisone 10 mg daily. In contrast to the 3-weekly regimen there was no survival benefit of the weekly schedule regimen compared with mitoxantrone and the side effect profile for the weekly compared to the 3-weekly schedule was not favourable apart from a lower incidence of neutropenia [70]. A smaller phase 3 trial randomised men with mCRPC to docetaxel 3-weekly versus a 2-weekly schedule (50 mg/m^2 d 1 and d 15, every 28 d). There was a small benefit for the 2-weekly schedule for the primary endpoint (time to treatment failure) as well as an improvement in OS and there was a lower rate of haematological toxicity for the 2-weekly schedule [79].

Regarding docetaxel chemotherapy for men with mCRPC there was a consensus (86%) that the 3-weekly regimen (75 mg/m^2) should be used, 10% voted for the 2-weekly (50 mg/m^2) schedule and 4% for a weekly schedule.

The FIRSTANA trial compared cabazitaxel 25 mg/m^2 to cabazitaxel 20 mg/m^2 and to docetaxel 75 mg/m^2 as first-line chemotherapy in men with mCRPC. The data were presented (ASCO 2016; NCT01308567) but are not published and did not show a significant difference in OS. The PROSELICA trial was also presented at ASCO 2016 and showed noninferiority for the primary endpoint of OS for cabazitaxel 20 mg/m^2 compared with cabazitaxel 25 mg/m^2 in men with mCRPC progressing on or after docetaxel (NCT01308580).

For cabazitaxel there was a consensus (79%) to start with the 20 mg/m^2 dose in the majority of patients, 59% of panellists use this dose (with dose reductions in subsequent cycles if indicated), 20% voted for starting with this dose and to escalate to 25 mg/m^2 in the absence of relevant side effects. Seventeen percent of the panellists voted for starting with a dose of 25 mg/m^2 in the majority of men.

In the subset of panellists who voted for cabazitaxel 25 mg/m^2, 57% of the panellists voted for the use of prophylactic WBC growth factors from start of therapy in the majority of patients, 26% voted for the use in a minority of selected patients, 8% voted for use of these growth factors only for marrow toxicity occurring beyond start of therapy, and 9% do not use them at all.

In the subset of panellists who voted for cabazitaxel 20 mg/m^2, 30% voted for prophylactic WBC growth factors from start of therapy in the majority of patients, 32% in a minority of selected patients, 27% only for marrow toxicity, and 11% did not vote for the use of growth factors.

Table 8 – What is your preferred choice between abiraterone and enzalutamide at any time in the treatment sequence in men with metastatic castration-resistant prostate cancer (mCRPC) if all options are available in case of the following medical situations?

What is your preferred choice between abiraterone and enzalutamide at any time in the treatment sequence in men with mCRPC if all options are available in case of the following medical situations?	Abiraterone (%)	Enzalutamide (%)	Either (%)	Neither: alternative treatment option preferred (%)	Abstain (%)
History of falls	94	2	4	0	0
Baseline significant fatigue	88	6	6	0	0
Baseline significant neurocognitive impairment	84	4	10	2	0
Stable brain metastases	73	6	10	11	0
Long QTc-syndrome or men on not replaceable drugs with potential QT prolongation	27	31	24	12	6
Asymptomatic men with a duration of response to ADT (no chemo-hormonal therapy) <12 mo	6	11	56	27	0
Cardiac ejection fraction below 45–50	6	63	27	2	2
Active liver dysfunction	8	66	14	12	0
Diabetes mellitus requiring prescription drug therapy	6	84	10	0	0

ADT = androgen deprivation therapy.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

5.3. Combination therapy for mCRPC

In mCRPC there are currently no combination treatment strategies for survival prolonging agents that have shown an OS benefit as compared with monotherapy. A number of large randomised phase 3 clinical trials combining abiraterone with enzalutamide or other novel endocrine agents and abiraterone or enzalutamide with radium-223 dichloride are currently ongoing (eg, NCT02194842M; NCT02043678; NCT01949337). The question of combination strategies is especially relevant for radium-223, because of the lack of antitumour activity outside the bone since soft tissue and visceral metastases are not uncommon in men with APC [80].

In men with symptomatic mCRPC and bone metastases, 18% of the panellists voted for the combination of radium-223 with either abiraterone or enzalutamide from the beginning as a first-line treatment for mCRPC for the majority of patients, 38% in a minority of selected patients, and 42% of the panellists did not vote for this combination.

In men with mCRPC being treated with abiraterone or enzalutamide for bone and soft tissue metastases and who are progressing only in the bone, 43% of the panellists voted for the addition of radium-223 to the majority of such patients, 39% in a minority of selected patients, and 18% did not vote for adding radium-223 in this situation.

In men with mCRPC treated with radium-223 and progressing outside of the bone 52% of the panellists voted for completing treatment with radium-223 plus adding abiraterone or enzalutamide (if they have not received either drug before) in the majority of patients, 20% in a minority of selected patients, and 26% did not vote for this approach.

5.4. Poor prognosis, aggressive variant mCRPC

While the majority of APCs remain driven by AR signalling, it has become increasingly recognized that a subset of mCRPC tumours may adapt during the course of therapy to become less dependent on the AR, and this is associated with loss of luminal prostate cancer markers (including PSA), the development of lineage plasticity, and the acquisition or expansion of small cell/neuroendocrine pathologic and molecular features [81,82]. Identification of mCRPC variants remains challenging but is often suspected in patients that develop rapidly progressive disease, unusual sites or pattern of metastases (eg, radiologically lytic bone or parenchymal brain metastases), and/or progression in the setting of a low and not or modestly rising PSA. Metastatic tumour biopsies in this setting may show small cell carcinoma, but are not always straightforward as mixed, atypical adenocarcinoma, and hybrid neuroendocrine phenotypes may also occur [82].

The votes of the panellists concerning factors for definition of poor prognosis, aggressive variant mCRPC are reported in Table 9. There was no consensus regarding the definition of poor prognosis, aggressive variant mCRPC. Four percent of the panellists did not believe poor prognosis, aggressive variant mCRPC is a clinically meaningful entity.

The publication of the olaparib data in heavily pretreated mCRPC patients with DNA repair defects in the absence of an approved poly (adenosine diphosphate-ribose) polymerase (PARP) inhibitor for mCRPC has revived the interest for the use of platinum-based chemotherapy regimens, especially in later lines. The combination of carboplatin and docetaxel has shown good antitumour activity in a nonrandomised phase 2 clinical trial with patients selected for poor prognosis features [83]. A randomised phase 2 trial of cabazitaxel plus carboplatin versus cabazitaxel alone has been presented but is not published and showed significantly improved antitumour activity with the combination treatment (NCT01505868, ASCO 2015).

Regarding first-line treatment of the majority of men with poor prognosis, aggressive variant (putting aside pure small cell carcinoma) 58% of the panellists voted for standard mCRPC treatment, 36% voted for a platinum- and taxane-based combination therapy, 4% for a platinum- and etoposide-based combination therapy, and 2% for a platinum monotherapy.

Table 9 – Which of the following criteria would you use to define poor prognosis, aggressive variant metastatic castration-resistant prostate cancer (mCRPC) putting aside pure small cell prostate cancer?

Which of the following criteria would you use to define poor prognosis, aggressive variant mCRPC putting aside pure small cell prostate cancer:	Yes (%)	Only in combination with other unfavourable factors (%)	No (%)	I do not believe poor prognosis, aggressive variant mCRPC is a clinically meaningful entity (%)	Abstain (%)
Neuro-endocrine differentiation on a tumour biopsy and/or low or absent androgen receptor expression	71	27	0	2	0
Exclusive visceral metastases	70	20	6	4	0
Rapid progression without correlation with PSA kinetics	63	31	4	2	0
Low PSA levels relative to tumour burden	45	47	6	2	0
Predominantly lytic bone metastases	45	39	14	2	0
Short response to androgen deprivation therapy (≤12 mo) for metastatic prostate cancer	34	60	4	2	0
Bulky tumour masses	21	65	12	2	0

PSA = prostate-specific antigen.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

5.5. Monitoring in men with mCRPC treated with radium-223

The phase 3 radium-223 trial (ALSYMPCA) enrolled patients with symptomatic mCRPC [72]. Patients were randomised to six injections of radium-223 administered every 4 wk or to best standard of care alone. OS was improved in the intent to treat analysis for patients randomized to radium-223 [72]. Substantial declines in PSA and/or lactate dehydrogenase were uncommon in both arms. However, alkaline phosphatase (ALP) levels showed a decline in the radium treated patients with 87% of radium treated patients showing some decline in ALP at wk 12 [84].

In the subset of panellists who use radium-223 in men with mCRPC 43% voted for testing of PSA every cycle, 43% for every 2–4 mo; 8% voted for PSA testing only if clinically indicated, and 6% for no PSA testing in this situation.

Regarding ALP testing these panellists voted for either every cycle (49%) or every 2–4 mo (37%). Eight percent voted for ALP testing only if clinically indicated and 6% voted for no ALP testing.

Since the ALSYMPCA trial did not mandate any imaging for response monitoring, the role of imaging in men treated with radium-223 is not well documented. Symptomatic and PSA flares after radium-223 have been described and can be accompanied by bone scintigraphy flare [85]. Early changes in bone scintigraphy and CT assessments tend to be unreliable for bone response assessment and must thus be interpreted with caution. In a retrospective series of 130 men treated with radium-223 that had baseline imaging and monitoring by imaging after three and six cycles, the results showed a significant rate of progression outside of the bone detected by CT scanning [85].

In the subset of panellists who use radium-223 in men with mCRPC there was consensus (75%) to use CT and bone scintigraphy for staging and monitoring of men on radium-223, while 23% of the panellists voted for one of the next-generation imaging methods. Regarding imaging frequency for men treated with radium-223, 41% of these panellists voted for every 3–4 mo, 27% voted for imaging after 6 mo (completion of radium-223) and every 3–4 mo thereafter, 24% voted for imaging after 6 mo (completion of radium-223) and follow-up imaging at progression, 4% voted for imaging only as clinically indicated.

5.6. "Oligo-progressive" mCRPC

With the introduction of abiraterone and enzalutamide as first-line treatment for asymptomatic men with mCRPC, there are men in whom, for example, a single lymph node progresses in size with radiological stability of the other lesions. The term oligo-progressive is not well-defined in APC but in lung cancer patients on novel targeted agents such as anaplastic lymphoma kinase (ALK) inhibitors there is growing literature on definition and treatment strategies for oligo-progressive disease [86].

There was no consensus as to the most meaningful definition of oligo-progressive prostate cancer (mCRPC). Forty percent of the panel voted that they did not believe in oligo-progressive disease as a meaningful clinical entity, 33% voted for the definition of only one progressing pre-existing lesion with otherwise stable/responding metastatic disease, 23% voted for ≤3 progressing pre-existing lesions with otherwise stable/responding metastatic disease.

The subset of the panel who believed in oligo-progressive mCRPC voted on biopsy of a progressing lesion (for diagnostic purposes). Twenty-nine percent of the panellists voted for a biopsy in the majority of patients, 52% for a biopsy in a minority of selected patients (eg, from visceral metastases), while 19% did not vote for a biopsy. These panellists also voted on the treatment for men with oligo-progressive mCRPC: 40% voted for a change or addition of systemic therapy without local treatment, 47% for local treatment of the progressing lesion(s) while continuing systemic therapy unchanged, and 13% for local treatment of the progressing lesion(s) plus adding or changing the systemic treatment.

5.7. Discussion of CRPC

We have witnessed the successful development of agents including the novel androgen signalling inhibitors abiraterone and enzalutamide for earlier stage mCRPC. More recently, a significant survival advantage by introducing docetaxel treatment in the castration-naive state was confirmed. It thus appears that we are moving our therapies earlier in the disease, while the question of optimal sequencing of the treatment options is still unanswered. We know that a distinct subset of patients will not respond to treatment also depending on the sequence, or may experience unwarranted toxicity. Moreover, it is possible that with the appropriate sequencing we may augment the OS benefit of our patients.

Treatment sequencing in APC is governed by a number of parameters that unfortunately do not yet serve the ultimate goal of maximizing clinical outcome. Clinical decision-making is still largely dependent on local reimbursement policies and on a number of variables that are not truly objective. There are no validated clinical or molecular predictive markers for guiding our choice thus predetermining a more favourable cost/benefit ratio for our patients. Increased benefit is encompassing longer life with improved quality whereas minimising cost including components such as toxicity, financial burden, and uncertainty. Choices made in the clinic are in part based on objective data such as available level I evidence and access to agents. Yet, professional speciality and experience affect these choices. The presence or absence of symptoms clearly influenced treatment selection for the panellists.

We are also being challenged by the as-yet unproven hypothesis that combinatorial approaches may enhance outcome by potential synergistic activity or delay of resistance to treatment. We are anticipating results from several relevant phase 3 trials and should therefore avoid implementation of such approaches as long as they are unproven especially since concerns for toxicity arise.

Regarding the aggressive variant of CRPC, the majority of the panel recognises its existence and that it is important to recognise it since these patients may be less likely to respond to subsequent AR-directed therapies; however,

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

there was no consensus for the exact definition. With a more profound and eventually earlier suppression of AR pathways in the disease history, identifying and treating AR independent variants will become increasingly important [87]. The development of robust biomarkers is an area of active research. We may need a combination of clinical and molecular features to identify aggressive variants, encompassing but not limited to those with neuroendocrine carcinoma morphology detected on biopsy, as targeted treatment approaches based on a molecular subclassification of APC are developed. Understanding the role of DNA repair in contributing to the phenotype, mediating response to PARP inhibition, and also platinum sensitivity and potential immunotherapy treatment sensitivity is also important.

6. Imaging in APC

Reproducible and validated methods for detecting and quantifying metastatic disease are needed to manage patients with APC. Currently, recommended methods of metastatic imaging assessment, that is, with bone scintigraphy and CT scans, have significant limitations in detecting metastases as well as in monitoring response to treatment but remain the standard of care in most settings [1,21,88–91]. Due to limitations in systematically conducted prospective studies, the use of next-generation imaging has not been shown to impact on clinical outcome.

6.1. Nodal disease assessments in APC

Morphologic assessments for possible nodal disease using CT and MRI scans are based on the evaluation of detected nodes based largely on size criteria. Other morphologic criteria, such as the nodal shape, loss of nodal hilum fat, clustering, extranodal disease, and enhancement characteristics can serve as additional aids to diagnosis. Unfortunately, morphologic imaging is unable to identify micrometastases or to distinguish large hyperplastic benign from malignant nodes. Thus, the general test performance of morphologic imaging remains limited when histologic correlations using template lymphadenectomy are used as the standard of reference. A meta-analysis showed a CT scan sensitivity of 42% and specificity of 82%, while morphologic MRI had a sensitivity of 39% and a specificity of 82% [92]. While positron emission tomography (PET)/CT has improved sensitivity, it is important to keep in mind that the spatial resolution of PET/CT is approximately 4 mm.

6.2. Bone disease assessments in APC

For the sensitive detection of metastatic bone disease, the use of current recommendation of bone scintigraphy and CT scans has low sensitivity and specificity [93].

Systematic analyses, prospective clinical studies, and meta-analyses have shown comparative test performance of whole-body diffusion weighted MRI (WB-MRI) to NaF and choline PET/CT for the skeletal assessments in APC [94,95]. A recent meta-analysis underlined the usefulness of WB-MRI as a method that improves the MRI detection of bone metastases [96]. When evaluating the results of the above meta-analyses and indeed in all studies reporting test performance, the readers should note that there are intrinsic verification biases that are particularly prevalent at lesion level analyses, because it is not possible to obtain histopathology for every bone lesion detected. As a result, most studies are patient level analyses, using combinations of imaging methods and/or follow-up as the standards of reference [93,94].

PET/CT can detect a larger number of skeletal lesions than bone scintigraphy [97]. Regarding the PET/CT tracers comparative studies between prostate-specific membrane antigen (PSMA) and choline have demonstrated superiority of PSMA to identify bone lesions [98]. The PET tracer ^{18}F-fluciclovine has recently been approved for use in North America; available data indicate good detection rates both for lymph nodes and for bone disease in biochemical recurrence of prostate cancer [99]. The diagnostic performance of fluciclovine PET was found to be superior to CT and to choline PET but there are no comparative data versus WB-MRI and PSMA PET [100].

Importantly, all our prognostic models and clinical trials in APC were developed using CT scan and bone scintigraphy and the essence of detection of disease at diagnosis is one of risk determination. Next generation imaging may have superior performance characteristics compared with older modalities, but clinical validation with regard to the question of impact on outcome has not yet been performed.

6.3. Imaging for locally advanced prostate cancer

In men presenting with high-risk or locally advanced prostate cancer and with biochemical recurrence after local therapy, imaging to document potential metastases may be important. At this state of the disease metastases are most commonly located within regional (N1) and nonregional lymph nodes as well as in bone (M1).

There was no consensus regarding the imaging modality to "exclude" distant metastases in high-risk and locally advanced prostate cancer: 41% of the panel voted for a combination of CT and bone scintigraphy, while 47% of the panel voted for next-generation imaging methods (37% voted for a PET/CT with any of the tracers PSMA, choline, or fluciclovine and 10% voted for a WB-MRI).

6.4. Imaging in the setting of BCR (PSA)

Clinical symptoms and PSA alone are not good indicators for absence of metastases, with 32% of clinical M0 CRPC patients being found to be metastatic when imaging was performed [101].

Regarding PET/CT in BCR, a meta-analysis including both C-11 and F-18 choline-based techniques reported detection rates greater than 50% for PSA values above 2 ng/ml, with rapid PSA kinetics and elevated Gleason score positively related to higher detection rates [102–105]. The main limitation of choline PET/CT is the low sensitivity when PSA values are <1 ng/ml. In BCR there are comparative studies

between Ga-PSMA and choline demonstrating the superiority of Ga-PSMA in terms of detection rates at any PSA level [106–108]. Guidelines (NCCN, EAU) have mentioned choline PET/CT in the situation of BCR [21,22].

The use of next-generation imaging modalities has led to identification of metastatic foci at lower PSA levels. Treating physicians may feel more comfortable offering ablation of limited metastases in these cases, but as of now there are no prospective data to show that earlier detection of metastatic disease with next-generation imaging results in a meaningful long-term clinical improvement.

Imaging in men with rising PSA after RP before starting SRT was voted for by 44% of the panellists in the majority of patients independent of PSA level, by 29% of panellists in men with a PSA >0.5 ng/ml, by 12% of the panellists in men with a PSA >1 ng/ml and by 13% of the panellists in men with a PSA >2 ng/ml.

For imaging in men with oligometastatic recurrent disease after local treatment for prostate cancer with curative intent (± SRT), 78% of the subset of panellists who believed in the oligometastatic recurrent state voted for one of the next-generation imaging methods to detect metastatic disease: namely 47% voted for a PET/CT (PSMA, choline, or fluciclovine) alone, 2% voted for a WB-MRI alone, 25% of the panel members voted for a combination of a pelvic MRI and a PET/CT, 4% of the panellists voted for a combination of a pelvic MRI and a WB-MRI, and 22% of the panellists voted for imaging by CT and/or MRI and bone scintigraphy.

In men with de novo apparent oligometastatic disease, 72% of the subset panellists who believed in the oligometastatic state voted for one of the next-generation imaging methods to support this diagnosis (apart from local staging): namely 34% voted for a PET/CT (PSMA, choline, or fluciclovine), 4% voted for a WB-MRI, 34% voted for either a PET/CT or WB-MRI, and 26% of these panellists voted for imaging by CT and/or MRI and bone scintigraphy.

Asked about the recommended tracer in case of a PET/CT in men with apparent oligometastatic castration-naïve disease, there was a consensus (76%) amongst the panel members for PSMA as tracer, 10% voted for fluciclovine as a tracer, and 6% voted for choline; 4% of the panellists voted for any of the three tracers.

In men with rising PSA on ADT (CRPC) and potentially oligometastatic disease, 74% of the subset of panellists who believe in oligometastatic disease in mCRPC voted for one of the next-generation imaging methods to confirm this diagnosis: namely 48% voted for a PET/CT (PSMA, choline, or fluciclovine), 6% voted for a WB-MRI, 18% of the panel members voted for a combination of a pelvic MRI and a PET/CT, 2% of the panellists voted for a combination of a pelvic MRI and a WB-MRI, and 26% of the panellists voted for imaging by CT and/or MRI and bone scintigraphy.

6.5. Staging and monitoring in mCNCP

In mCNPC, what is required is an imaging modality that confirms the presence of metastases and defines their location. This is important for assessing prognosis and for treatment decisions. Current guidelines (NCCN, EAU) do not comment on imaging methods for men with mCNPC because of lack of data.

In mCNPC, 51% of the panel voted for baseline imaging and follow-up imaging at PSA nadir/completion of six cycles of docetaxel as part of chemo-hormonal therapy and again at progression (confirmed PSA rise and/or clinical progression), 31% of the panel voted for baseline imaging and regular monitoring by imaging every 3–6 mo, and 18% of the panel voted for baseline imaging only and monitoring by PSA alone with further imaging at progression.

Regarding the recommended imaging modality for staging and monitoring of men with mCNPC, 73% of the panel voted for CT and bone scintigraphy and 25% of the panellists voted for one of the next-generation imaging methods.

6.6. Staging and monitoring in mCRPC

The early identification of treatment failure in men with mCRPC on systemic therapy would help in sparing some patients futile treatment and potential toxicity as well as in reducing the costs of ineffective treatments and decreasing the time to initiation of a next-line, potentially effective treatment [110]. Recent data indicate that there are a substantial number of patients who have radiographic progression without PSA progression, including some patients with aggressive variant prostate cancer [111]. Imaging before treatment initiation and on-therapy may be important in predicting both benefit and more importantly nonbenefit of treatments.

An ideal imaging method to monitor response to therapy should enable the evaluation of tumour cell viability, especially for bone disease. Techniques such as bone scintigraphy, CT scans, and NaF PET rely on tumour matrix interactions and are only indirect indicators of tumour cell viability. Imaging assessments should always be combined with clinical status and other factors as also recommended by the PCWG3 group [109].

For monitoring by imaging in men with mCRPC on first-line therapy, 54% of the panel voted for baseline imaging and regular monitoring by imaging every 3–6 mo, 28% of the panellists voted for baseline imaging and follow-up imaging at PSA nadir and again at progression (confirmed PSA rise and/or clinical progression); 16% of the panel voted for baseline imaging only and monitoring by PSA alone with further imaging at progression.

Regarding imaging modality for staging and monitoring in men with mCRPC, 74% of the panel voted for CT and bone scintigraphy and 24% of the panellists voted for one of the next-generation imaging methods.

For monitoring of patients with a diagnosis of aggressive variant mCRPC, 62% of the panellists voted for standard imaging by CT and bone scintigraphy, 2% voted for CT alone, and 36% voted for next-generation imaging modalities.

6.7. Discussion of imaging in APC

There are sufficient data indicating that next-generation imaging technologies have better accuracy for detecting metastases than CT and bone scintigraphy. However, their

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

current use is dependent on costs, local availability, and expertise of interpretation and the better accuracy has not been shown to correlate with improvement of clinical outcomes.

The performance of PET/CT with new tracers (PSMA and fluciclovine) as indicators of treatment efficacy and as predictors of patient outcome has yet to be assessed. PSMA PET/CT should be interpreted with caution since there are data suggesting correlation between PSMA expression and AR signalling [112–117]. Tumour foci not expressing PSMA (or lesions in organs with high PSMA expression, eg, liver) may not be assessable for response using PSMA PET/CT. Notably, other tumour types (eg, lung cancer, renal cell cancer) and nonmalignant processes like Paget's disease and haemangioma can express PSMA [118,119].

The use of these next-generation imaging modalities may be especially valuable in situations where the tumour burden assessments are needed for treatment decisions and/or when high sensitivity is a requirement. This may be particularly applicable when multimodality salvage therapy is being considered. However, the proof that their use leads to better treatment decisions and ultimately leads to improved outcomes is pending also in this situation.

For evaluation of response in men with mCRPC it is evident that next-generation imaging (MRI and PET) may prove to be more accurate for evaluating response to treatment [120]. However, it should be noted that the recently published PCWG3 do not recommend the routine use of next-generation imaging methods for men with APC treated on clinical trials mainly due to the lack of availability, outcome data, and standardisation across global sites [109]. The recently published guideline on reporting WB-MRI in men with APC is a step into the right direction but these recommendations need to be adopted, applied, and validated in clinical trials with primary endpoint of clinical outcome [88]. As an example, the systematic evaluation of FDG-PET studies in patients with Hodgkin's disease has resulted in a reduction in treatment intensity leading to reduction of toxicity [121]. Such trials with next-generation imaging are largely missing in men with APC [122].

The clinical introduction of potentially impactful imaging technologies has created an opportunity for progress by linking anatomy to underlying biology but there is also a risk of up-staging of many men in every disease state. The contribution of the next-generation imaging techniques to the welfare of patients depends on performance for the purpose they are being applied (''fit for use'') and their clinical utility (patient benefit). The early assessment of new technologies is therefore encouraged but their general acceptance before measures of performance and evidence of benefit are at least estimated should not be supported. Novel imaging techniques should be clinically deployed ideally in a trial setting but at least in registries with the goal of efficiently estimating performance and utility. Finally, it is important to recognise that the clinical trials that form the basis of the currently approved treatment options are based on evaluations with CT and bone scintigraphy.

7. Use of osteoclast-targeted therapy for SRE/SSE prevention for mCRPC (not for osteoporosis/bone loss)

In prostate cancer, two bone-directed agents, zoledronic acid and denosumab have been shown to prevent or delay the onset of SREs. Neither of the drugs influences OS or PFS significantly [123,124].

Of the bisphosphonates, zoledronic acid is the only one that has shown a protective effect against SRE in patients with mCRPC [124,125]. Denosumab is a fully human monoclonal antibody that specifically targets receptor activator of nuclear factor kappa-B ligand thus effectively inhibiting osteoclast function and bone resorption. In the setting of mCRPC, denosumab (120 mg subcutaneous every 4 wk) compared with zoledronic acid (4 mg intravenous every 4 wk) significantly improved the time to first SRE [123].

At the present time, these agents have proven relevant efficacy only in patients with bone mCRPC. There is no evidence to support their use in the nonmetastatic CRPC setting and there is evidence **not** to use it in the mCNPC setting apart from osteoporosis prevention, using a different regimen, and dosage for both drugs [43,126,127].

When looking at SSE, two prospective randomised studies in men with mCRPC demonstrated an advantage. The TRAPEZE study showed a significant delay in SSEs when docetaxel was combined with zoledronic acid as compared with docetaxel alone and that the combination was safe, but there was no improvement in OS [128]. Interestingly, the benefit in delaying SSEs was in the same range as what was seen in the pivotal zoledronic acid study when chemotherapy was not in use. Also, the recent analysis of the large pivotal denosumab trial confirmed a benefit in preventing SSEs [129]. Hypothesis-generating results have been presented from the ALSYMPCA trial where the subgroup of patients receiving a combination of radium-223 plus an osteoclast targeted therapy had a reduction in SSE compared with radium-223 alone [72,130].

In an era of life prolonging therapies for mCRPC that can also prevent or delay SREs, the added benefit of osteoclast-targeted therapy is difficult to estimate given the limited number of well designed, adequately powered studies with long term follow-up.

Regarding the frequency of administration of these bone-directed agents a recent randomised trial in different tumour types also including 689 men with prostate cancer showed no increased risk of skeletal events with zoledronic acid every 12 wk compared with every 4 wk [131]. However, the proportion of patients with CNPC versus CRPC is not reported and both were accrued to the trial. No firm conclusions can be made from this trial because of this variable.

For reducing the risk of skeletal complications in men with mCRPC and bone metastases, 86% of the panel were in favour of some form of osteoclast-targeted therapy, 54% of the panel voted for denosumab, 8% voted for zoledronic acid, 24% of the panellists voted for either zoledronic acid or denosumab, and 10% did not vote for an osteoclast-targeted therapy at all.

Of those panellists who voted for an osteoclast-targeted therapy in men with mCRPC, 68% voted for a treatment

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

duration of about 2 yr and 32% voted for no limitation of treatment duration.

The question of frequency and duration of osteoclast-targeted therapy in the absence of significant toxicity for asymptomatic men with mCRPC and bone metastases responding to first-line systemic mCRPC treatment is not resolved.

In the subset of panellists who voted for osteoclast-targeted therapy in men responding to first-line mCRPC therapy, 17% of the panellists voted for every 4 wk without a defined maximum duration, 37% voted for every 4 wk for approximately 2 yr and then less frequently, 15% voted for every 3 mo, and 27% of the panel did not vote for an osteoclast-targeted therapy in this situation. In the same patient population, but when these men are no longer responding to first-line therapy, 27% of the panellists voted for osteoclast-targeted therapy every 4 wk without a defined maximum duration and 53% of the panel voted for every 4 wk for about 2 yr and then less frequently.

Osteonecrosis of the jaw (ONJ) is a possible severe side effect of osteoclast-targeted therapy that increases with the duration of treatment [132,133]

In men with mCRPC who develop ONJ while on osteoclast-targeted therapy, there was consensus (84%) to discontinue osteoclast-targeted therapy permanently while 16% of the panellists voted for discontinuation of the osteoclast-targeted therapy and restarting after complete wound healing.

7.1. Discussion of the use of osteoclast-targeted therapy for SRE/SSE prevention for mCRPC

The optimal timing, schedule, and duration for osteoclast-targeted therapy and the overall balance of benefit and risk as well as efficacy in the era of novel mCRPC treatments are still a matter of debate as there is no Level I evidence to guide decision making.

Effective osteoclast inhibitors are commonly recommended as part of the overall therapeutic approach to mCRPC also in an era of multiple life prolonging agents. Their use in combination with approved life prolonging mCRPC treatments may enhance their utility in terms of reducing the risk for of skeletal complications and to maintain quality of life—but these data have been derived from posthoc and subgroup analyses and need to be addressed in prospective clinical trials. In daily clinical practice, the risk of side effects—especially ONJ—which increases with duration of therapy, by the early use of osteoclast-targeted therapy for men with mCRPC has to be weighed up against the potential benefit of reduction in risk of SRE/SSE [133].

8. Molecular characterisation

8.1. Tumour biopsy in APC

Since clinical heterogeneity is common, mCRPC tumour biopsies should be reviewed and interpreted in the appropriate clinical context. This is especially important for uncommon yet challenging cases with small cell or neuroendocrine differentiation or tumours that lack expression of classical prostate markers such as PSA or AR. Furthermore, not all patients with clinical features suggestive of androgen independence demonstrate small cell or neuroendocrine features on tumour biopsy although they may still benefit from platinum based chemotherapy. These data may potentially be explained by molecular overlap with neuroendocrine prostate cancer [81,134].

Moving forward, incorporating molecular biomarkers will likely improve the clinical diagnosis of non-AR driven mCRPC and may help in patient selection for current therapies and selection for biomarker stratified clinical trials [134–140]. Genomic alterations enriched in mCRPC with emerging prognostic and/or treatment implications include *AR* gene mutation and amplification, phosphoinositide 3-kinase/Akt/phosphatase and tensin homolog pathway alterations, DNA repair defects including loss of homologous recombination (eg, BRCA1/2, ATM), and mismatch repair (with microsatellite instability [MSI] and hyper-mutated phenotype), TP53 deletion/mutation, and RB1 loss [134,140–144]. Alterations involving RB1 and TP53 are universal in small cell cancers arising elsewhere in the body, such as lung cancer, and are enriched in prostate cancer patients with luminal to basal cell lineage switching and neuroendocrine biomarker expression and are mechanistically involved in the development of "androgen indifferent" resistance [136,139,140,143].

The panel voted on molecular factors that should be reported in a tumour biopsy in men with mCRPC apart from reporting tumour morphology (Table 10).

There was a consensus (78%) that BRCA1, BRCA2, and ATM mutations should be reported because that knowledge will likely influence management decisions. For all other factors there was no consensus (Table 10).

8.2. Androgen receptor splice variant-7 and AR amplification/mutation

Using liquid biopsies in mCRPC patients starting abiraterone or enzalutamide, statistically significant associations with worse outcome have been reported for detection of AR splice variants including the AR-V7 transcripts in circulating cells or in exosomes, AR-V7 protein in the circulating tumor cell nucleus, or by analysing plasma cell-free DNA *AR* gene copy number gain assessed via cell-free DNA or somatic point mutations similarly quantified [145–150]. All studies to date were single-arm trials, and statistically significant associations with response were noted—although the correlation with response has focused largely on rates of PSA declines. Moreover, evidence remains that some men with AR-V7 positive mCRPC may still respond to abiraterone/enzalutamide.

There was a consensus (96%) not to use AR-V7 testing in daily routine clinical practice for the majority of men with mCRPC. Similarly, there was a consensus (92%) not to use cell-free DNA AR amplification and AR mutation testing in daily routine clinical practice for the majority of men with mCRPC.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

Table 10 – As a clinician, which factors do you want to have reported back to you in men with metastatic castration-resistant prostate cancer who undergo a metastatic tumour biopsy apart from tumour morphology and differentiation? The question is only about management for a specific patient, not about familial implications, and based on knowledge in terms of test accuracy/validity and available treatments

Factor	Yes, useful test for majority of patients (influences your management decision; %)	Only for minority of selected patients (%)	No (%)	Abstain (%)
BRCA1, BRCA2, and ATM mutations	78	20	2	0
PSA IHC	72	18	10	0
Other DNA repair genes (eg, *CHEK2*, *PALB2*, and others)	64	22	12	2
MMR gene alterations (MSI, MMR protein IHC, or by direct sequencing)	54	22	20	4
Chromogranin, synaptophysin, CD56/NSE	50	31	17	2
Loss of PTEN	44	26	26	4
AR amplification and/or AR mutation	43	18	37	2
TP53 and RB1	34	22	40	4
Nuclear AR	34	18	46	2
AR-V7	33	26	37	4
PSMA	32	22	44	2
Ki67/MiB1	28	26	42	4
Prostate acid phosphatase	26	18	54	2
PD-1/PD-L1	22	31	45	2
NKX3.1	12	33	49	6
ERG IHC	12	30	56	2
ERG FISH	11	23	64	2

AR = androgen receptor; FISH = fluorescent in situ hybridization; IHC = immunohistochemistry; MMR = mismatch repair; MSI = microsatellite instability; PD-1 = programmed cell death-1; PD-L1 = programmed death-ligand 1; PSA = prostate-specific antigen; PSMA = prostate-specific membrane antigen; PTEN = phosphatase and tensin homolog.

8.3. Somatic mutations

Recent genomic studies of metastatic prostate cancer have identified new molecular targets in the AR signalling pathway, phosphoinositide 3-kinase pathway, WNT pathway, cell cycle pathways, and perhaps most importantly, in DNA repair pathways [135,141,151].

Fifty-nine percent of the panellists did not vote for DNA sequencing of tumour biopsies in the majority of men with mCRPC in routine daily clinical practice, 37% of the panellists voted for a targeted/panel sequencing approach, and 4% voted for whole genome or exome sequencing.

8.4. DNA repair testing in daily routine clinical practice

Recent studies have shown that men with APC commonly have somatic aberrations of genes that make up various elements of the DNA repair machinery with 20–30% of APCs having loss of function of proteins implicated in homologous recombination repair, including *BRCA2*, *BRCA1*, *ATM*, *PALB2*, and others [141]. These aberrations lead to homologous recombination deficiency (HRD) detectable by next-generation sequencing of these genes or of the genomic scars resulting from this repair defect estimated as an HRD score. A clinical trial (TOPARP) of the PARP inhibitor, olaparib, has shown antitumour activity against prostate cancers with HRD [142].

HRD defects have been previously reported to sensitise tumour cells to platinum-based chemotherapy [152]. Clinical data are now emerging that HRD defects in prostate cancers also sensitise to platinum-based chemotherapy [153] in keeping with previous reports that satraplatin has antitumour activity against this disease [76,154].

Somatic deleterious aberrations of mismatch repair genes (*MSH2*, *MSH6*, *MLH1*, *PMS2*) have been found in APC, and are possibly associated with ductal pathology, although their precise frequency remains uncertain and is in the range of 5% to 15% [144,155,156].

8.4.1. DNA repair defects in CNPC

The presence of DNA repair defects (germline or somatic) in men with newly diagnosed mCNPC does not change the standard treatment recommendation for 49% of the panel. Twenty-three percent of the panellists were more likely to give docetaxel in addition to ADT and 22% of the panel were more likely to include a platinum agent in the chemo-hormonal treatment regimen.

8.4.2. DNA repair defects in mCRPC

When testing for DNA repair defects was considered for men with mCRPC, and no recent mCRPC tissue biopsy tissue was available, 70% of the subset of panellists who supported testing in this situation voted for a fresh mCRPC tumour biopsy, 16% of the panellists voted for testing in archival tissue, and 14% voted for testing in circulating cell-free DNA.

Sixty-five percent of the panel voted for treatment with olaparib, or another PARP inhibitor if available and approved, in men with mCRPC and the presence with DNA repair defects (germline or somatic) based on the phase 2 data with olaparib, 29% of the panel voted for such treatment in a minority of selected patients and 4% did not vote for it at all.

Some panel members voted that it was appropriate to extrapolate the phase 2 data from olaparib to platinum agents for men with mCRPC and presence of DNA repair defects (germline or somatic): 45% in the majority of patients and 14% in a minority of selected patients; however 35% of the panellists did not support this extrapolation.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

Sixty-seven percent of the panel voted for standard first-line mCRPC therapy in men with mCRPC and presence of DNA repair defects (germline or somatic) progressing on ADT, 21% of the panellists voted for a platinum-based combination, and 10% for a PARP inhibitor.

In men with mCRPC and a presence of DNA repair defects in the second-line setting (after standard first-line therapy), 40% of the panellists voted for a platinum-based combination, 33% of the panel voted for standard second-line mCRPC treatment, 21% for treatment with a PARP-inhibitor, and 4% for a platinum monotherapy.

8.5. Discussion of molecular characterisation

Given men with mCRPC are surviving longer, and with several treatment options available, biopsies of metastatic lesions are more commonly pursued to rule out small cell carcinoma, an aggressive variant, or a second malignancy. But the real place for metastases biopsy remains unclear in everyday practice. With a multitude of potential predictive and prognostic markers that can be tested in a mCRPC tumour biopsy, it is important to provide some guidance. As of March 2017, there was only consensus from the panel for testing of *BRCA1*, *BRCA2*, and *ATM* mutations in mCRPC tissue.

Several registration trials are now being conducted with different PARP inhibitors for men with APC and evidence of DNA repair defects (eg, NCT02952534, NCT02975934, NCT02854436, NCT03012321) and in the absence of approved PARP inhibitors for mCRPC, enrolment of men in clinical trials is strongly recommended.

Additionally, there are also prospective trials of platinum-based therapy ongoing in men with advanced molecularly selected prostate cancers, which may demonstrate that this is an important therapeutic strategy for this subgroup of patients (eg, NCT02598895, NCT02311764, NCT02955082).

Although true MSI is rare in prostate cancer, its presence is important because MSI+ cancers have a high rate of durable responses to immune checkpoint blockade using drugs that block the programmed cell death-1/programmed death-ligand 1 interaction [157]. Based on 149 patients with MSI-H or dMMR cancers enrolled across five uncontrolled, multi-cohort, multi-center, single-arm clinical trials pembrolizumab has been approved by the FDA for use in MSI high and dMMR cancer patients regardless of histology. This approval is of clear interest to clinicians and to patients with prostate cancer and evidence of these alterations.

Although a proportion of the panel voted for using a PARP inhibitor or platinum-based chemotherapy in mCRPC, even in the first-line setting, there is no evidence that such a strategy is of advantage as compared with the standard approved mCRPC treatments to date. Therefore, in the absence of prospective randomised trials showing clinical benefit for a strategy using a PARP-inhibitor or a platinum-based chemotherapy, the use of these substances as first-line mCRPC treatment outside of clinical trials should not be generally recommended.

For the liquid biomarkers, namely AR-V7 and AR mutation or amplification, there was a consensus that currently none of these markers should be tested in routine practice for decision making. This consensus against testing is in part based upon the low detection levels of AR-V7 prior to first- and second-line therapies and the high probability that patients would receive abiraterone or enzalutamide in this situation. These tests need to be validated and further studies need to be performed to determine their impact on long-term outcomes.

9. Germline genetic counselling/testing

The aetiology of prostate cancer is not well understood, although epidemiological studies demonstrating a convergence of incidence rates in some populations migrating between areas with a low incidence to those with high incidence suggest environmental and lifestyle risk factors play a role [158]. Having a positive family history and/or a certain ethnic background such as Afro-Caribbean is a risk factor for prostate cancer development. Evidence from studies where monozygotic twins were compared with dizygotic twins suggest that 57% of the risk of prostate cancer prostate cancer is due to genetic factors [159]. Numerous studies of risks to relatives of prostate cancer cases show a higher relative risk of developing prostate cancer, which increases as the age of the proband decreases, and the number of affected relatives increases. First degree relatives of prostate cancer patients have twice the risk of developing the disease compared with the general population [160]. In men diagnosed under the age of 60 yr, the risk to their first degree relatives is more than fourfold that of those without a family history [161]. The variation in incidence according to ethnicity also suggests a genetic component; rates are higher in African American men compared with Asian-American men [162].

Studies of familial inheritance and segregation analyses have proposed various genetic models (autosomal dominant, recessive, and X-linked) [163]. It is now recognised that genetic predisposition to prostate cancer is composed of common (>5%) lower risk variants single nucleotide polymorphisms—most of which are not in coding regions and rare higher risk variants (coding mutations in genes). Over 100 single nucleotide polymorphisms associated with the development of prostate cancer have been identified thus far [164].

Rarer variants are those which have a minor allele frequency of <5%, and occur too infrequently to be detected on a genome-wide association study. Next-generation sequencing of targeted areas or whole genome/exome sequencing has enabled the detection of these rare variants. Results showed that men from families where females had developed breast and ovarian cancer caused by *BRCA* mutations have a five-fold relative risk of prostate cancer when they harbour a germline *BRCA2* mutation compared with men without a mutation. This relative risk increases to up to seven-fold if the men in the family develop prostate cancer below the age of 65 yr [165]. In a larger study, 2000 men with prostate cancer were screened. This showed

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

that just over 1% of men who developed prostate cancer below the age of 65 yr carried a deleterious *BRCA2* mutation and often they did not have a positive family history [166]. For men who are carriers of a *BRCA1* mutation, studies have shown that there is an approximately four-times relative risk of developing prostate cancer for men aged under 65 yr compared with those without the mutation [167]. It has been subsequently shown in men with a family history of at least three cases of prostate cancer that they have a germline mutation in DNA repair genes in 7.3% and that the disease was more likely to be aggressive [168].

Several groups have shown that *BRCA1* and *BRCA2* mutation carriers have a more aggressive form of prostate cancer and also have a worse prognosis [169,170]. Mutation carriers are also likely to present with a higher risk of local nodal involvement as well as with distant metastatic disease [171]. The optimal radical treatment option for these patients is yet to be determined, but RP may be the most suitable, although the numbers of patients studied are relatively small [172].

Remarkably, germline mutations have been found in about half of the men with tumour HR DNA repair gene defects and about one in five men with an mismatch repair DNA repair gene defect [141,173]. In a large multi-institutional study of almost 700 men with metastatic prostate cancer unselected for age or family history, 11.8% overall were found to have moderate or high penetrance germline mutations in one of 16 DNA repair genes, with 7.8% of mutations in *BRCA2*, *BRCA1*, and *ATM* [173]. Two large single-institution studies of metastatic prostate cancer found similar rates of germline *BRCA2*, *BRCA1*, and *ATM* mutations, with much lower rates in low risk indolent disease [174,175].

Regarding genetic counselling and testing for men with newly diagnosed metastatic prostate cancer, 20% of the panel voted to do it in a majority of patients: 62% of the panel voted in favour of genetic counselling/testing in a minority of selected patients and 18% did not vote to do it at all.

The subset of panellists who had voted for genetic testing in a minority of selected patients supported genetic counselling and testing in men with a positive family history for prostate cancer (95%); also, 93% of these panellists supported counselling/testing in men with a positive family history for other cancer syndromes (eg, hereditary breast and ovarian cancer syndrome and/or pancreatic cancer or Lynch syndrome). Further, 74% of these panellists voted for genetic counselling and testing in men with prostate cancer diagnosed at ≤60 yr but 26% of these panellists did not vote for genetic counselling and testing based on an age cut-off alone.

Among the subset of panellists who recommended genetic testing, 61% voted for large panel testing including homologous recombination and mismatch DNA repair (eg, comprehensive cancer risk assessment panels), 15% voted for BRCA1 and BRCA2 testing only, 15% voted for BRCA1, BRCA2, and ATM testing, and 9% voted for large panel testing including homologous recombination DNA repair (eg, panels that are also used to assess breast cancer risk).

There was a consensus (92%) that in the presence of a germline BRCA1, BRCA2, or ATM mutation a prophylactic RP was ***not*** *recommended.*

The panel was asked whether the presence of a germline BRCA1, BRCA2, or ATM mutation would influence their treatment decision in men with low-risk localised prostate cancer. Forty-five percent voted against active surveillance in these patients, 35% voted for standard treatment options (including active surveillance), and 20% voted for another treatment option.

The panel was asked whether the presence of a germline BRCA1, BRCA2, or ATM mutation would influence their treatment decision in men with intermediate- or high-risk localised prostate cancer. Fifty-two percent of the panel voted for a RP over RT, 44% of the panel voted for standard recommendations, and 4% voted for RT over a RP.

9.1. Discussion of germline genetic counselling/testing

The understanding of the role of genetics in prostate cancer development is evolving rapidly, which is reflected by the fact that 20% of the panellists recommended genetic counselling and testing in a majority of men with metastatic prostate cancer irrespective of family history. Age at diagnosis itself does not seem to be the best selection marker, but 74% of the panel who recommended genetic counselling and testing in selected patients would test in men aged ≤60 yr. The impact of a *BRCA2* germline mutation on the management in an otherwise healthy man is not clear and in the absence of any prospective data there was a consensus not to recommend prophylactic RP in such men.

Currently, for prostate cancer care providers ordering germline genetic cancer panel testing or ordering this testing in the near future, there are several important points to consider including which genes to test for. There are emerging prostate cancer practice recommendations only for *BRCA1*, *BRCA2*, and *ATM* mutations, yet most next-generation sequencing cancer panels include many more DNA repair genes for the same cost. There are currently no gene-specific data on treatment predication or prostate cancer risk for most DNA repair genes. Germline genetic testing should be ordered with adequate pretest and/or posttest genetic counselling. In particular, there is a need to counsel about the possibility of a variant of uncertain significance (VUS) being detected and/or a pathogenic mutation in a gene in which there are not adequate data to alter management for prostate cancer. Patients with VUS should be managed the same as patients with a negative test result, and there is a danger that in daily practice VUS may be misinterpreted as a positive result. The question of testing of family members is unanswered and screening recommendations if mutations are detected need to be generated. There are data suggesting earlier PSA screening in men with *BRCA2* and potentially also in men with *BRCA1* germline mutations [176]. More data are needed to appropriate counsel unaffected male family members about prostate cancer risk and make screening recommendations.

Large collaborative efforts are underway (eg, NCT00261456, PRACTICAL consortium) to address some of the open questions. However, in order to move the field forward more efforts are needed to collaborate—especially on prostate cancers with germline mutations that occur at a

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

low frequency. The panel recommends to be especially careful (not overinterpret) about treatment recommendations based on germline mutations in men with localised prostate cancer.

10. Side effects of systemic treatment: prevention, management, and supportive care

A substantial proportion of men with APC will die of a noncancer-related cause and must live with the acute and chronic side effects of treatment. Most men with localised prostate cancer do not die of their disease, but will spend the rest of their lives managing the effects of the treatment they have undergone. The wishes of our patients and their families are clear: they wish to be cured of their disease or to have their survival prolonged, but not necessarily at the cost of intolerable side effects of treatment. Sometimes it is easy to lose sight of this goal in the search for better oncological outcomes.

One-hundred percent of the panel believed that there was at least moderate evidence that ADT increases the risk of bone loss and/or fractures; 87% believed this evidence was strong.

Baseline measurement of vitamin D for men with prostate cancer starting on ADT was voted for in the majority of patients by 43% of the panellists, in a minority of patients by 26% and 31% of the panellists did not vote for it.

Routine supplementation of calcium and vitamin D for men with prostate cancer starting on ADT was voted for by 73% of the panel, only of vitamin D by 13%, only calcium by 2%, and 12% of the panel did not vote for routine supplementation.

A baseline measurement of bone mineral density in men with prostate cancer starting on ADT was voted for by 62% of the panellists in the majority of patients, by 15% only in patients with nonmetastatic disease and 21% did not vote for it at all.

Drug therapy to prevent bone loss and/or fractures with denosumab or a bisphosphonate in the dose and schedule for osteoporosis prophylaxis in men with prostate cancer starting on ADT was voted for in the majority of patients by 16% of the panellists, by 70% of panellists only in patients with documented osteopenia or osteoporosis, and 12% did not vote for it.

Thirty-five percent of the panellists felt that there is strong evidence that ADT increases the risk of diabetes, 46% felt that there is moderate, and 17% that there is weak evidence for this correlation. Two percent believe that ADT does not change the risk of diabetes.

For cardiovascular disease, 12% of the panellists felt that there is strong evidence that ADT increases the risk, 39% felt that there is moderate, and 45% that there is weak evidence for this correlation. Four percent believe that ADT does not change the risk of cardiovascular disease.

A history of recent/severe cardiovascular disease influenced the choice of ADT in men with metastatic prostate cancer for 29% of the panellists in the majority of patients, for 41% of the panellists for a minority of selected patients, and for 28% of the panellists it did not influence their choice of ADT.

For the subset of panellists whose decisions was influenced by a history of recent/severe cardiovascular disease, 11% voted for using LHRH agonists, 52% for use of LHRH antagonists, 6% for orchiectomy, 20% for any form of intermittent ADT, and 11% voted for bicalutamide 150 mg/d in such a patient.

Eight percent of the panellists believed that there is strong evidence that ADT increases the risk of cognitive changes and/or dementia, 29% felt that there is moderate, and 50% that there is weak evidence for this correlation. Thirteen percent believe that ADT does not change the risk of cognitive changes and/or dementia.

For depression, 6% of the panellists believed that there is strong evidence that ADT increases the risk, 46% felt that there is moderate, and 44% that there is weak evidence for this correlation. Four percent believe that ADT does not change the risk of depression.

A multidisciplinary management team can include the necessary expertise to deal with these issues [177]. Improved outcomes are apparent with involvement of prostate cancer nurses and care coordinators. Endocrinologists and andrologists can provide advice on the management of diabetes, metabolic syndrome, bone health, cardiovascular, and sexual health. Psychologists can provide support for the common problems of suicidal risk, distress, and long-term psychological and sexual morbidity [178–181]. The exercise physiologist can provide programs to counteract the effects of ADT, improve psychological symptoms, and improve overall and disease-specific survival [182–184]. The direct provider of care for men with APC can also learn such skills.

Comprehensive geriatric assessment has been shown to be associated with a higher probability of completing a treatment course, fewer modifications of treatment, and lower toxicity [185,186].

Routine involvement of a multidisciplinary/multiprofessional team for prevention or management of ADT related adverse effects was voted for by 42% of the panellists for the majority of patients, by 39% in a minority of selected patients, and 17% did not vote for it.

Sixty-one percent of the panellists voted for early access to an expert in symptom palliation or a dedicated palliative care service and 39% of the panellists did not vote for it.

There was consensus (94% of the panellists) for access to opiate pain medication for men with metastatic prostate cancer and severe pain when lower level pain medication is not sufficient.

Thirty percent of the panellists voted for a health status assessment in men with APC $\geq$70 yr before treatment decision in the majority of patients, 42% voted for it in a minority of selected patients, and 24% did not vote for it.

The subset of panellists who voted for a health status assessment voted for comprehensive geriatric assessment in 26%, G8 and Mini-COG in 29%, G8 alone in 30%, and another tool in 15%.

There was consensus (98% of the panellists) for regular physical exercise in men with prostate cancer starting on ADT.

10.1. Discussion of side effects of systemic treatment: prevention, management, and supportive care

The aging population of men with APC is now surviving longer, allowing longer-term complications of treatment to

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

become apparent and to affect function and symptoms. The evidence that ADT negatively impacts bone health and the attendant risk for fractures is considered strong by a majority of the panel. ADT has also been associated with an increased risk of metabolic syndrome, type 2 diabetes, and sarcopenia; however, evidence linking ADT directly as a cause of vascular disease is weak and there is no convincing evidence that ADT is linked causally to the development of dementia as reflected in the vote of the panellists [187–196]. Men should be informed about the acute but also the long-term side effects of ADT and importantly the possible preventive measures.

Interestingly, there was no consensus for the routine assessment of health status in men aged 70 yr, likely based on the fact that there are no large prospective clinical trials which have shown that using health status assessment in men with metastatic prostate cancer has a relevant impact on outcome, especially when compared with the judgement of experienced physicians. This recommendation could also reflect a lack of consensus on what would constitute such a "health status assessment." Finally, there is a need for clinical trials and registration studies specifically in this patient population.

11. Global access to prostate cancer drugs and treatment in countries with limited resources

The panel voted on a number of questions regarding treatment options in men with APC in lower and middle-income countries (LMIC) because the topic of global access to APC treatments was discussed at APCCC 2017.

If living in a country with limited resources available for health care, 90% of the panellists voted for orchiectomy as ADT in the metastatic setting. The remaining 10% voted for an LHRH agonist.

As second-line endocrine manipulations in LMIC in men with mCRPC progressing on ADT, 44% of the panellists voted for a first generation AR antagonist, 24% for steroid monotherapy, 20% for ketoconazole, 8% for oestrogens, and 4% for estramustine.

Each of the following drugs is on the World Health Organization (WHO) essential medicines list and/or they can be sourced at an affordable price from generic manufacturer. The panel voted on appropriate treatment options in the setting of limited health care resources in men with mCRPC who are progressing on or after docetaxel: *77% of the panellists voted for a platinum, 19% did not vote for it. Mitoxantrone was voted for by 69% of the panellists. Thirty-nine percent voted for the use of cyclophosphamide, 53% did not. There was a consensus not to use paclitaxel (78%) or doxorubicin (84%) in this situation.*

11.1. Discussion of global access to prostate cancer drugs and treatment in countries with limited resources

Prostate cancer generally is more common in higher income countries, but this is changing as men in LMIC live longer, due to better control of infectious disease and other causes of early mortality. Men in LMIC tend to present with more advanced disease and access to the survival prolonging agents for mCRPC is limited for many men in LMIC.

Although the panel recommended orchiectomy as first choice of ADT in men presenting with metastatic prostate cancer, the socio-cultural and psychological barriers to such an intervention must be taken into consideration in such treatment decisions.

As secondary hormonal treatment option for men with mCRPC, endocrine manipulations including glucocorticoids, oestrogens, first generation androgen receptor inhibitors, and ketoconazole are available and the panel considered especially first-generation AR inhibitors a valid treatment option in LMIC.

Abiraterone and enzalutamide are examples of high-cost drugs with limited access in LMIC. Both drugs were developed substantially through research in academic laboratories and cancer centres. In the USA, approved doses are marketed at ~US$ 7000/mo, while publicly funded health systems such as Britain and Canada have been able to negotiate a substantially lower price of ~$3000/mo. Generic abiraterone (but not enzalutamide) is available in India for about $450/mo, which is, however, still too expensive for many men with mCRPC in India.

The following drugs which have shown some antitumour activity but no OS benefit in men with mCRPC and are on the WHO essentials medicine list: carboplatin, paclitaxel, doxorubicin, and cyclophosphamide. Carboplatin was recommended by a majority of the panellists. Mitoxantrone is not on the WHO essentials medicine list but has shown a pain palliation benefit and could be sourced at a reasonable price. Many of these drugs are substantially cheaper than the approved and survival prolonging agents for mCRPC and they can be used sometimes as substitutes for newer agents in LMIC. While this is a reasonable strategy, it falls far short of the ideal of providing the most effective treatments to all men with APC.

A major goal of this consensus conference is to improve the management and outcomes of men with APC. However, it is a suboptimal clinical achievement to show that new treatments can improve the duration and quality of survival of men with APC, but to have such treatments unavailable to a large segment of the global population of men with APC. The availability of RT as a very effective bone pain palliation therapy is not given in many countries. We cannot easily change the way that drugs are developed and marketed for profit by academic, pharmaceutical, and biotechnology companies, and we certainly respect and collaborate within this system for the development of needed new treatments for men with APC. But men with APC are still unable to access optimal treatments, oftentimes not because they could not be made available, but because they are not made available at an affordable price. Hence, we encourage ongoing multidisciplinary and stakeholder dialogue to further address this global issue.

12. Conclusions

In the absence of Level I evidence and in areas where there are conflicting data or conflicting interpretation of available

Areas of consensus (≥ 75% agreement) APCCC 2017

Management of high-risk localised and locally advanced prostate cancer

- Lymph node dissection in men with cN0 cM0 high-risk prostate cancer undergoing prostatectomy: 84%
- Minimal requirement for lymph node sampling in men with cN0 cM0 high-risk prostate cancer
  - Obturator lymph nodes: 98%
  - External iliac lymph nodes: 85%
  - Internal iliac lymph nodes: 90%
  - Not to sample paraaortic lymph nodes: 95%
- For pathology reporting in case of lymphadenectomy:
  - Number and anatomic region of resected lymph nodes and no. and location of involved lymph nodes: 94%
  - Micro- vs macrometastases: 81%
  - Metastatic deposits in perinodal fat tissue: 79%
  - Extranodal extension of involved lymph nodes: 81%
- Reporting of prostatectomy specimen in locally advanced prostate cancer:
  - Seminal vesicle involvement: 100%
  - Extent of prostatic involvement: 96%
  - Gleason score or grade group, extraprostatic extension, positive surgical margins: number length, and location, as well as grade at margin: 100%
  - Tertiary Gleason score: 94%

"Oligometastatic" prostate cancer

- If positron emission tomography–computed tomography is considered in oligometastatic castration-naïve prostate cancer (CNPC) prostate-specific membrane antigen as a tracer: 76%

Management of castration-naive prostate cancer

- Factors rendering a patient as "not being suitable for docetaxel":
  - Severe hepatic impairment: 96%
  - Neuropathy grade ≥2: 82%
  - Platelets $<50 \times 10^9$/l and/or neutrophils $<1.0 \times 10^9$/l: 81%
- Docetaxel in addition to androgen deprivation (ADT) therapy in CNPC
  - De novo metastatic CNCP and high-volume disease: 96%
  - Not to add docetaxel in biochemical relapse (N0 M0): 90%
- 3-weekly docetaxel (75 mg/m^2) regimen in CNPC: 96%

Management of castration-resistant prostate cancer (CRPC)

- First-line CRPC
  - Abiraterone or enzalutamide for asymptomatic men without docetaxel for CNPC: 86%
  - Abiraterone or enzalutamide for asymptomatic men with docetaxel for CNPC: 90%
  - Abiraterone or enzalutamide for asymptomatic men with docetaxel for CNPC and progressed within ≤6 mo after completion of docetaxel in the CNPC setting: 77%
  - Not to combine radium-223 and docetaxel: 88%
- Second-line CRPC
  - Taxane in men with symptomatic mCRP C who had progressive disease as best response to first-line abiraterone or enzalutamide: 96%
  - Taxane in men with symptomatic mCRPC and secondary (acquired) resistance (initial response followed by progression) after use of first-line abiraterone or enzalutamide: 90%
  - Abiraterone or enzalutamide for asymptomatic men with mCRPC progressing on or after docetaxel for mCRPC (without prior abiraterone or enzalutamide): 92%
  - Abiraterone or enzalutamide for symptomatic men with mCRPC progressing on or after docetaxel for mCRPC (without prior abiraterone or enzalutamide): 76%

Fig. 1 – Areas of consensus Advanced Prostate Cancer Consensus Conference (APCCC) 2017.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

- Preferred choice between abiraterone and enzalutamide in special situations:
  - Abiraterone in case of a history of falls: 94%
  - Abiraterone in case of baseline significant fatigue: 88%
  - Abiraterone in case of baseline significant neurocognitive impairment: 84%
  - Enzalutamide in case of diabetes mellitus requiring prescription drug therapy: 84%
- 3-weekly docetaxel (75 mg/m^2) in the CRPC setting: 86%

Imaging

- Computed tomography and bone scintigraphy for staging and treatment monitoring in men with mCRPC on treatment with radium-223: 75%

Osteoclast-targeted therapies

- Discontinuation of osteoclast targeted treatment in men who develop osteonecrosis of the jaw while on osteoclast-targeted therapy for skeletal related events/symptomatic skeletal events prevention: 84%

Molecular characterisation

- Tumour biopsy reporting in mCRPC
  - *BRCA1, BRCA2, and ATM* status: 78%
- Liquid biomarkers in routine clinical practice
  - Not to do androgen receptor (AR)-variant 7 testing: 96%
  - Not to do cell-free DNA AR amplification and AR mutation: 92%

Genetic counselling/testing

- Not to do a prophylactic prostatectomy in t he presence of a germline *BRCA1, BRCA2, or ATM* mutation: 92%

Side effects of systemic treatment and supportive care

- Advise patients about strong evidence that ADT increasesrisk of bone loss and/or fractures: 87%
- Regular physical exercise in men with prostate cancer starting on ADT: 98%
- Access to opiate pain medication for men with metastatic prostate cancer and severe pain when their lower level pain medication is not sufficient: 94%

Global access to prostate cancer drugs and treatment in countries with limited resources

- Orchiectomy as ADT in the metastatic setting: 90%
- In men with mCRPC who are progressing on or after docetaxel:
  - Platinum (carboplatin/cisplatin): 77%
  - Not paclitaxel: 78%
  - Not doxorubicin: 84%

Fig. 1. (*Continued*).

data, weighted expert opinions can be helpful for treatment decisions in daily routine clinical practice. It is important to note that expert opinion is not equivalent to high-level evidence and that current expert consensus may be disproven by future clinical research.

There were several notable areas of consensus in APCC 2017 as summarised in Figure 1.

There were also several notable areas of panellist disagreement including but not limited to: (1) chemo-hormonal therapy in "low-volume" CNPC, (2) treatment of the primary tumour in metastatic disease, (3) radium-223 combination strategies, (4) use of platinum in mCRPC, (5) definition of aggressive variant prostate cancer, (6) use, schedule, and duration of osteoclast-targeted therapies especially in the context of newer survival prolonging mCRPC therapies; (7) use of next-generation imaging; (8) how to advise men with known *BRCA2*, *BRCA1*, or *ATM* mutations; (9) adjuvant RT; (10) when to initiate SRT; (11) definition and treatment for oligometastatic synchronous and metachronous prostate cancer; (12) health status assessment in patients aged $\geq$70 yr; and (13) pathology reporting of men undergoing a mCRPC biopsy.

The panel members recognise that the voting results may contribute to the adoption of unproven or controversial interventions and interfere with prospective clinical research to evaluate the efficacy and safety of those

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

interventions. A problem arising from the widespread initiation of unvalidated techniques and treatments is that they achieve a clinical momentum, which makes it very difficult to conduct effective comparative studies. The panel strongly recommends participation in clinical research to inform clinical management with high-level evidence. Important research areas are adjuvant and salvage treatment; diagnosis and treatment of oligometastatic disease; molecular characterisation; personalised therapy strategies; and supportive care including the impact of geriatric assessment and specific interventions.

We urgently need public and/or charity funding to carry out studies in areas such as surgery, RT, or imaging where financial support from industry is commonly not available.

Additional relevant questions remain that we were not able to address in detail in this meeting such as costs and cost-effectiveness of drugs, health economic issues, and patient-reported outcomes. APCCC 2019 plans to address these questions and the above-mentioned areas of controversy and new emerging topics.

Author contributions: Silke Gillessen and Aurelius Omlin had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Not applicable.
Acquisition of data: All authors.
Analysis and interpretation of data: All authors.
Drafting of the manuscript: All authors.
Critical revision of the manuscript for important intellectual content: All authors.
Statistical analysis: None.
Obtaining funding: Gillessen, Omlin.
Administrative, technical, or material support: None.
Supervision: All authors.
Other: None.

Financial disclosures: All authors certify that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: Detailed conflict of interest statements for all panel members can be found in the Supplemental material.

Funding/Support and role of the sponsor: Not applicable.

Acknowledgments: We gratefully acknowledge all the participants in the Advanced Prostate Cancer Consensus Conference (APCCC) for their lively, stimulating discussions. We thank Thomas Cerny for his support and Stefanie Fischer for carefully reading and correcting the manuscript.
We gratefully acknowledge the financial support of the following nonprofit organisations for the APCCC 2017: City and Canton of St. Gallen, Swiss Cancer Research Organisation, European School of Oncology, Swiss Cancer League, the Swiss Oncology Research Network SAKK, Swiss Cancer Foundation, Prostate Cancer Foundation. We would like to thank especially the Movember Foundation for generously supporting the APCCC 2017 conference.
IDD is supported by an NHMRC Practitioner Fellowship (APP1102604). We would also like to thank the following supporters: Cantonal Hospital St. Gallen, EMPA Materials Science and Technology, Appenzeller Bier, Egger Gemüsebau, Moving light and sound, Schwyter, Swiss, Wernli.
We also acknowledge sponsorship from several different for-profit organisations including: Astellas, Bayer Health Care, Janssen, Sanofi Genzyme, Amgen, Bristol-Myers Squibb, Ferring, Roche, Tolmar, AstraZeneca, Clovis, Medivation, Orion, and Uro-Today (details on www.apccc.org). These for-profit organisations supported the conference financially but had no input in the scientific content or the final publication.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2017.06.002.

References

[1] Gillessen S, Omlin A, Attard G, et al. Management of patients with advanced prostate cancer: recommendations of the St Gallen Advanced Prostate Cancer Consensus Conference (APCCC) 2015. Ann Oncol 2015;26:1589–604.
[2] Diamond IR, Grant RC, Feldman BM, et al. Defining consensus: a systematic review recommends methodologic criteria for reporting of Delphi studies. J Clin Epidemiol 2014;67:401–9.
[3] James ND, Spears MR, Clarke NW, et al. Failure-free survival and radiotherapy in patients with newly diagnosed nonmetastatic prostate cancer: data from patients in the control arm of the STAMPEDE trial. JAMA Oncol 2016;2:348–57.
[4] Tombal B, Alcaraz A, James N, Valdagni R, Irani J. Can we improve the definition of high-risk, hormone naive, non-metastatic prostate cancer? BJU Int 2014;113:189–99.
[5] Mottet N, Bellmunt J, Bolla M, et al. EAU-ESTRO-SIOG Guidelines on Prostate Cancer. Part 1: screening, diagnosis, and local treatment with curative intent. Eur Urol 2017;71:618–29.
[6] Buyyounouski MK, Choyke PL, McKenney JK, et al. Prostate cancer—major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin 2017;67:245–53.
[7] Epstein JI, Amin MB, Reuter VE, Humphrey PA. Contemporary Gleason Grading of Prostatic Carcinoma: an update with discussion on practical issues to implement the 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. Am J Surg Pathol 2017;41:e1–7.
[8] Moch H, Humphrey PA, Ulbright TM. WHO Classification of Tumours of the Urinary System and Male Genital Organs. ed 4. Lyon, France: IARC; 2016.
[9] Passoni NM, Fajkovic H, Xylinas E, et al. Prognosis of patients with pelvic lymph node (LN) metastasis after radical prostatectomy: value of extranodal extension and size of the largest LN metastasis. BJU Int 2014;114:503–10.
[10] Briganti A, Larcher A, Abdollah F, et al. Updated nomogram predicting lymph node invasion in patients with prostate cancer undergoing extended pelvic lymph node dissection: the essential importance of percentage of positive cores. Eur Urol 2012;61:480–7.
[11] Dell'Oglio P, Abdollah F, Suardi N, et al. External validation of the European association of urology recommendations for pelvic lymph node dissection in patients treated with robot-assisted radical prostatectomy. J Endourol 2014;28:416–23.
[12] Hinev AI, Anakievski D, Kolev NH, Hadjiev VI. Validation of nomograms predicting lymph node involvement in patients with prostate cancer undergoing extended pelvic lymph node dissection. Urol Int 2014;92:300–5.

[13] Moul JW, Sarno MJ, McDermed JE, Triebell MT, Reynolds MA. NADiA ProsVue prostate-specific antigen slope, CAPRA-S, and prostate cancer-specific survival after radical prostatectomy. Urology 2014;84:1427–32.
[14] Bolla M, van Poppel H, Tombal B, et al. Postoperative radiotherapy after radical prostatectomy for high-risk prostate cancer: long-term results of a randomised controlled trial (EORTC trial 22911). Lancet 2012;380:2018–27.
[15] Wiegel T, Bartkowiak D, Bottke D, et al. Adjuvant radiotherapy versus wait-and-see after radical prostatectomy: 10-year follow-up of the ARO 96–02/AUO AP 09/95 Trial. Eur Urol 2014;66:243–50.
[16] Thompson IM, Tangen CM, Paradelo J, et al. Adjuvant radiotherapy for pathological T3N0M0 prostate cancer significantly reduces risk of metastases and improves survival: long-term followup of a randomized clinical trial. J Urol 2009;181:956–62.
[17] Roach 3rd M, Thomas K. Overview of randomized controlled treatment trials for clinically localized prostate cancer: implications for active surveillance and the United States preventative task force report on screening? J Natl Cancer Inst Monogr 2012;2012:221–9.
[18] Gandaglia G, Briganti A, Clarke N, et al. Adjuvant and salvage radiotherapy after radical prostatectomy in prostate cancer patients. Eur Urol. In press. http://dx.doi.org/10.1016/j.eururo.2017.01.039.
[19] Abdollah F, Gandaglia G, Suardi N, et al. More extensive pelvic lymph node dissection improves survival in patients with node-positive prostate cancer. Eur Urol 2015;67:212–9.
[20] Briganti A, Wiegel T, Joniau S, et al. Early salvage radiation therapy does not compromise cancer control in patients with pT3N0 prostate cancer after radical prostatectomy: results of a match-controlled multi-institutional analysis. Eur Urol 2012;62:472–87.
[21] Cornford P, Bellmunt J, Bolla M, et al. EAU-ESTRO-SIOG Guidelines on Prostate Cancer. Part II: treatment of relapsing, metastatic, and castration-resistant prostate cancer. Eur Urol 2017;71:630–42.
[22] National Comprehensive Cancer Network. Prostate Cancer. 2017. https://www.nccn.org/professionals/physician_gls/f_guidelines.asp.
[23] Tendulkar RD, Agrawal S, Gao T, et al. Contemporary update of a multi-institutional predictive nomogram for salvage radiotherapy after radical prostatectomy. J Clin Oncol. In press. http://dx.doi.org/10.1200/JCO.2016.67.9647.
[24] Abugharib A, Jackson WC, Tumati V, et al. Very early salvage radiotherapy improves distant metastasis-free survival. J Urol 2017;197:662–8.
[25] Carrie C, Hasbini A, de Laroche G, et al. Salvage radiotherapy with or without short-term hormone therapy for rising prostate-specific antigen concentration after radical prostatectomy (GETUG-AFU 16): a randomised, multicentre, open-label phase 3 trial. Lancet Oncol 2016;17:747–56.
[26] Shipley WU, Seiferheld W, Lukka HR, et al. Radiation with or without antiandrogen therapy in recurrent prostate cancer. N Engl J Med 2017;376:417–28.
[27] Zaffuto E, Gandaglia G, Fossati N, et al. Early postoperative radiotherapy is associated with worse functional outcomes in patients with prostate cancer. J Urol 2017;197:669–75.
[28] Shaikh MP, Alite F, Wu MJ, Solanki AA, Harkenrider MM. Adjuvant radiotherapy versus wait-and-see strategy for pathologic T3 or margin-positive prostate cancer: a meta-analysis. Am J Clin Oncol. In press. http://dx.doi.org/10.1097/COC.0000000000000358.
[29] Bolla M, Van Tienhoven G, Warde P, et al. External irradiation with or without long-term androgen suppression for prostate cancer with high metastatic risk: 10-year results of an EORTC randomised study. Lancet Oncol 2010;11:1066–73.
[30] Bolla M, Collette L, Blank L, et al. Long-term results with immediate androgen suppression and external irradiation in patients with locally advanced prostate cancer (an EORTC study): a phase III randomised trial. Lancet 2002;360:103–6.
[31] Roach 3rd M, Bae K, Speight J, et al. Short-term neoadjuvant androgen deprivation therapy and external-beam radiotherapy for locally advanced prostate cancer: long-term results of RTOG 8610. J Clin Oncol 2008;26:585–91.
[32] Pilepich MV, Caplan R, Byhardt RW, et al. Phase III trial of androgen suppression using goserelin in unfavorable-prognosis carcinoma of the prostate treated with definitive radiotherapy: report of Radiation Therapy Oncology Group Protocol 85-31. J Clin Oncol 1997;15:1013–21.
[33] Pilepich MV, Krall JM, al-Sarraf M, et al. Androgen deprivation with radiation therapy compared with radiation therapy alone for locally advanced prostatic carcinoma: a randomized comparative trial of the Radiation Therapy Oncology Group. Urology 1995;45:616–23.
[34] Hellman S, Weichselbaum RR. Oligometastases. J Clin Oncol 1995;13:8–10.
[35] Tosoian JJ, Gorin MA, Ross AE, Pienta KJ, Tran PT, Schaeffer EM. Oligometastatic prostate cancer: definitions, clinical outcomes, and treatment considerations. Nat Rev Urol 2017;14:15–25.
[36] Reyes DK, Pienta KJ. The biology and treatment of oligometastatic cancer. Oncotarget 2015;6:8491–524.
[37] Ost P, Bossi A, Decaestecker K, et al. Metastasis-directed therapy of regional and distant recurrences after curative treatment of prostate cancer: a systematic review of the literature. Eur Urol 2015;67:852–63.
[38] Ploussard G, Almeras C, Briganti A, et al. Management of node only recurrence after primary local treatment for prostate cancer: a systematic review of the literature. J Urol 2015;194:983–8.
[39] Leuprolide Study G. Leuprolide versus diethylstilbestrol for metastatic prostate cancer. N Engl J Med 1984;311:1281–6.
[40] Klotz L, Vesprini D, Sethukavalan P, et al. Long-term follow-up of a large active surveillance cohort of patients with prostate cancer. J Clin Oncol 2015;33:272–7.
[41] Fizazi K, Lesaunier F, Delva R, et al. A phase III trial of docetaxel-estramustine in high-risk localised prostate cancer: a planned analysis of response, toxicity and quality of life in the GETUG 12 trial. Eur J Cancer 2012;48:209–17.
[42] Fizazi K, Faivre L, Lesaunier F, et al. Androgen deprivation therapy plus docetaxel and estramustine versus androgen deprivation therapy alone for high-risk localised prostate cancer (GETUG 12): a phase 3 randomised controlled trial. Lancet Oncol 2015;16:787–94.
[43] James ND, Sydes MR, Clarke NW, et al. Addition of docetaxel, zoledronic acid, or both to first-line long-term hormone therapy in prostate cancer (STAMPEDE): survival results from an adaptive, multiarm, multistage, platform randomised controlled trial. Lancet 2016;387:1163–77.
[44] Vale CL, Burdett S, Rydzewska LH, et al. Addition of docetaxel or bisphosphonates to standard of care in men with localised or metastatic, hormone-sensitive prostate cancer: a systematic review and meta-analyses of aggregate data. Lancet Oncol 2016;17:243–56.
[45] Maximum androgen blockade in advanced prostate cancer: an overview of the randomised trials. Prostate Cancer Trialists' Collaborative Group. Lancet 2000;355:1491–8.
[46] James ND, Spears MR, Clarke NW, et al. Survival with newly diagnosed metastatic prostate cancer in the "docetaxel era": data from 917 patients in the control arm of the STAMPEDE Trial (MRC PR08, CRUK/06/019). Eur Urol 2015;67:1028–38.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

[47] Gravis G, Fizazi K, Joly F, et al. Androgen-deprivation therapy alone or with docetaxel in non-castrate metastatic prostate cancer (GETUG-AFU 15): a randomised, open-label, phase 3 trial. Lancet Oncol 2013;14:149–58.
[48] Gravis G, Boher JM, Fizazi K, et al. Prognostic factors for survival in noncastrate metastatic prostate cancer: validation of the glass model and development of a novel simplified prognostic model. Eur Urol 2015;68:196–204.
[49] Sweeney CJ, Chen YH, Carducci M, et al. Chemohormonal therapy in metastatic hormone-sensitive prostate cancer. N Engl J Med 2015;373:737–46.
[50] Gravis G, Boher JM, Joly F, et al. Androgen deprivation therapy (ADT) plus docetaxel versus ADT alone in metastatic non castrate prostate cancer: impact of metastatic burden and long-term survival analysis of the randomized phase 3 GETUG-AFU15 trial. Eur Urol 2016;70:256–62.
[51] Kawano M, Mabuchi S, Matsumoto Y, et al. The significance of G-CSF expression and myeloid-derived suppressor cells in the chemoresistance of uterine cervical cancer. Sci Rep 2015;5:18217.
[52] Waight JD, Hu Q, Miller A, Liu S, Abrams SI. Tumor-derived G-CSF facilitates neoplastic growth through a granulocytic myeloid-derived suppressor cell-dependent mechanism. PloS One 2011;6:e27690.
[53] Welte T, Kim IS, Tian L, et al. Oncogenic mTOR signalling recruits myeloid-derived suppressor cells to promote tumour initiation. Nat Cell Biol 2016;18:632–44.
[54] Patrikidou A, Brureau L, Casenave J, et al. Locoregional symptoms in patients with de novo metastatic prostate cancer: morbidity, management, and disease outcome. Urol Oncol 2015;33:202 e9–17.
[55] Won AC, Gurney H, Marx G, De Souza P, Patel MI. Primary treatment of the prostate improves local palliation in men who ultimately develop castrate-resistant prostate cancer. BJU Int 2013;112:E250–5.
[56] Culp SH, Schellhammer PF, Williams MB. Might men diagnosed with metastatic prostate cancer benefit from definitive treatment of the primary tumor? A SEER-based study. Eur Urol 2014;65:1058–66.
[57] Rusthoven CG, Jones BL, Flaig TW, et al. Improved survival with prostate radiation in addition to androgen deprivation therapy for men with newly diagnosed metastatic prostate cancer. J Clin Oncol 2016;34:2835–42.
[58] Loppenberg B, Dalela D, Karabon P, et al. The Impact of local treatment on overall survival in patients with metastatic prostate cancer on diagnosis: a national cancer data base analysis. Eur Urol. In press. http://dx.doi.org/10.1016/j.eururo.2016.04.031.
[59] Engel J, Bastian PJ, Baur H, et al. Survival benefit of radical prostatectomy in lymph node-positive patients with prostate cancer. Eur Urol 2010;57:754–61.
[60] Lin CC, Gray PJ, Jemal A, Efstathiou JA. Androgen deprivation with or without radiation therapy for clinically node-positive prostate cancer. J Natl Cancer Inst 2015 May 9;107(7). pii: djv119. http://dx.doi.org/10.1093/jnci/djv119
[61] Polkinghorn WR, Parker JS, Lee MX, et al. Androgen receptor signaling regulates DNA repair in prostate cancers. Cancer Discov 2013;3:1245–53.
[62] Komura K, Jeong SH, Hinohara K, et al. Resistance to docetaxel in prostate cancer is associated with androgen receptor activation and loss of KDM5D expression. Proc Natl Acad Sci U S A 2016;113:6259–64.
[63] Stuchbery R, McCoy PJ, Hovens CM, Corcoran NM. Androgen synthesis in prostate cancer: do all roads lead to Rome? Nat Rev Urol 2017 Jan;14(1):49–58. http://dx.doi.org/10.1038/nrurol.2016.221.
[64] James N, de Bono JS, Spears MR, et al. Abiraterone for Prostate Cancer Not Previously Treated with Hormone Therapy. N Engl J Med 2017. http://dx.doi.org/10.1056/NEJMoa1702900.
[65] Fizazi N, Tran N, Fein L, et al. Abiraterone plus Prednisone in Metastatic, Castration-Sensitive Prostate Cancer. N Engl J Med 2017. http://dx.doi.org/10.1056/NEJMoa1704174.
[66] Badwe R, Hawaldar R, Nair N, et al. Locoregional treatment versus no treatment of the primary tumour in metastatic breast cancer: an open-label randomised controlled trial. Lancet Oncol 2015;16:1380–8.
[67] Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 2010;363:411–22.
[68] Ryan CJ, Smith MR, de Bono JS, et al. Abiraterone in metastatic prostate cancer without previous chemotherapy. N Engl J Med 2013;368:138–48.
[69] Beer TM, Armstrong AJ, Rathkopf DE, et al. Enzalutamide in metastatic prostate cancer before chemotherapy. N Engl J Med 2014;371:424–33.
[70] Tannock IF, de Wit R, Berry WR, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 2004;351:1502–12.
[71] Petrylak DP, Tangen CM, Hussain MH, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 2004;351:1513–20.
[72] Parker C, Nilsson S, Heinrich D, et al. Alpha emitter radium-223 and survival in metastatic prostate cancer. N Engl J Med 2013;369:213–23.
[73] de Bono JS, Logothetis CJ, Molina A, et al. Abiraterone and increased survival in metastatic prostate cancer. N Engl J Med 2011;364:1995–2005.
[74] de Bono JS, Oudard S, Ozguroglu M, et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. Lancet 2010;376:1147–54.
[75] Scher HI, Fizazi K, Saad F, et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. N Engl J Med 2012;367:1187–97.
[76] Hager S, Ackermann CJ, Joerger M, Gillessen S, Omlin A. Anti-tumour activity of platinum compounds in advanced prostate cancer-a systematic literature review. Ann Oncol 2016;27:975–84.
[77] Lorente D, Mateo J, Zafeiriou Z, et al. Switching and withdrawing hormonal agents for castration-resistant prostate cancer. Nat Rev Urol 2015;12:37–47.
[78] Lorente D, Omlin A, Ferraldeschi R, et al. Tumour responses following a steroid switch from prednisone to dexamethasone in castration-resistant prostate cancer patients progressing on abiraterone. Br J Cancer 2014;111:2248–53.
[79] Kellokumpu-Lehtinen PL, Harmenberg U, Joensuu T, et al. 2-Weekly versus 3-weekly docetaxel to treat castration-resistant advanced prostate cancer: a randomised, phase 3 trial. Lancet Oncol 2013;14:117–24.
[80] Pezaro CJ, Omlin A, Lorente D, et al. Visceral disease in castration-resistant prostate cancer. Eur Urol 2014;65:270–3.
[81] Beltran H, Tomlins S, Aparicio A, et al. Aggressive variants of castration-resistant prostate cancer. Clin Cancer Res 2014;20:2846–50.
[82] Watson PA, Arora VK, Sawyers CL. Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. Nat Rev Cancer 2015;15:701–11.
[83] Aparicio AM, Harzstark AL, Corn PG, et al. Platinum-based chemotherapy for variant castrate-resistant prostate cancer. Clin Cancer Res 2013;19:3621–30.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

[84] Sartor O, Coleman RE, Nilsson S, et al. An exploratory analysis of alkaline phosphatase, lactate dehydrogenase, and prostate-specific antigen dynamics in the phase 3 ALSYMPCA trial with radium-223. Ann Oncol 2017;28:1090–7.
[85] Keizman D, Fosboel MO, Reichegger H, et al. Imaging response during therapy with radium-223 for castration-resistant prostate cancer with bone metastases-analysis of an international multicenter database. Prostate Cancer Prostatic Dis. In press. http://dx.doi.org/10.1038/pcan.2017.6.
[86] Basler L, Kroeze SG, Guckenberger M. SBRT for oligoprogressive oncogene addicted NSCLC. Lung Cancer 2017;106:50–7.
[87] Epstein JI, Amin MB, Beltran H, et al. Proposed morphologic classification of prostate cancer with neuroendocrine differentiation. Am J Surg Pathol 2014;38:756–67.
[88] Padhani AR, Lecouvet FE, Tunariu N, et al. METastasis Reporting and Data System for Prostate Cancer: practical guidelines for acquisition, interpretation, and reporting of whole-body magnetic resonance imaging-based evaluations of multiorgan involvement in advanced prostate cancer. Eur Urol 2017;71:81–92.
[89] Gillessen S, Fanti S, Omlin A. Reply to the letter to the editor 'management of patients with advanced prostate cancer: recommendations of the St Gallen Advanced Prostate Cancer Consensus Conference (APCCC) 2015' by Gillessen et al. Ann Oncol 2015;26:2354–5.
[90] Bjurlin MA, Rosenkrantz AB, Beltran LS, Raad RA, Taneja SS. Imaging and evaluation of patients with high-risk prostate cancer. Nat Rev Urol 2015;12:617–28.
[91] Rozet F, Roumeguere T, Spahn M, Beyersdorff D, Hammerer P. Non-metastatic castrate-resistant prostate cancer: a call for improved guidance on clinical management. World J Urol 2016;34:1505–13.
[92] Hovels AM, Heesakkers RA, Adang EM, et al. The diagnostic accuracy of CT and MRI in the staging of pelvic lymph nodes in patients with prostate cancer: a meta-analysis. Clin Radiol 2008;63:387–95.
[93] Lecouvet FE, El Mouedden J, Collette L, et al. Can whole-body magnetic resonance imaging with diffusion-weighted imaging replace Tc 99m bone scanning and computed tomography for single-step detection of metastases in patients with high-risk prostate cancer? Eur Urol 2012;62:68–75.
[94] Jambor I, Kuisma A, Ramadan S, et al. Prospective evaluation of planar bone scintigraphy, SPECT, SPECT/CT, 18F-NaF PET/CT and whole body 1.5T MRI, including DWI, for the detection of bone metastases in high risk breast and prostate cancer patients: SKELETA clinical trial. Acta Oncol 2016;55:59–67.
[95] Shen G, Deng H, Hu S, Jia Z. Comparison of choline-PET/CT, MRI, SPECT, and bone scintigraphy in the diagnosis of bone metastases in patients with prostate cancer: a meta-analysis. Skeletal Radiol 2014;43:1503–13.
[96] Woo S, Suh CH, Kim SY, Cho JY, Kim SH. Diagnostic performance of magnetic resonance imaging for the detection of bone metastasis in prostate cancer: a systematic review and meta-analysis. Eur Urol. http://dx.doi.org/10.1016/j.eururo.2017.03.042.
[97] Minamimoto R, Loening A, Jamali M, et al. Prospective comparison of 99mTc-MDP scintigraphy, combined 18F-NaF and 18F-FDG PET/CT, and whole-body MRI in patients with breast and prostate cancer. J Nucl Med 2015;56:1862–8.
[98] Schwenck J, Rempp H, Reischl G, et al. Comparison of 68Ga-labelled PSMA-11 and 11C-choline in the detection of prostate cancer metastases by PET/CT. Eur J Nucl Med Mol Imaging 2017;44:92–101.
[99] Bach-Gansmo T, Nanni C, Nieh PT, et al. Multisite experience of the safety, detection rate and diagnostic performance of fluciclovine (18F) positron emission tomography/computerized tomography imaging in the staging of biochemically recurrent prostate cancer. J Urol 2017;197:676–83.
[100] Nanni C, Zanoni L, Pultrone C, et al. (18)F-FACBC (anti1-amino-3-(18)F-fluorocyclobutane-1-carboxylic acid) versus (11)C-choline PET/CT in prostate cancer relapse: results of a prospective trial. Eur J Nucl Med Mol Imaging 2016;43:1601–10.
[101] Yu EY, Miller K, Nelson J, et al. Detection of previously unidentified metastatic disease as a leading cause of screening failure in a phase III trial of zibotentan versus placebo in patients with nonmetastatic, castration resistant prostate cancer. J Urolo 2012;188:103–9.
[102] Evangelista L, Zattoni F, Guttilla A, et al. Choline PET or PET/CT and biochemical relapse of prostate cancer: a systematic review and meta-analysis. Clin Nucl Med 2013;38:305–14.
[103] Colombie M, Campion L, Bailly C, et al. Prognostic value of metabolic parameters and clinical impact of (1)(8)F-fluorocholine PET/CT in biochemical recurrent prostate cancer. Eur J Nucl Med Mol Imaging 2015;42:1784–93.
[104] Giovacchini G, Incerti E, Mapelli P, et al. [(1)(1)C]Choline PET/CT predicts survival in hormone-naive prostate cancer patients with biochemical failure after radical prostatectomy. Eur J Nucl Med Mol Imaging 2015;42:877–84.
[105] Pfister D, Porres D, Heidenreich A, et al. Detection of recurrent prostate cancer lesions before salvage lymphadenectomy is more accurate with (68)Ga-PSMA-HBED-CC than with (18)F-Fluoroethylcholine PET/CT. Eur J Nucl Med Mol Imaging 2016;43:1410–7.
[106] Morigi JJ, Stricker PD, van Leeuwen PJ, et al. Prospective comparison of 18F-fluoromethylcholine versus 68Ga-PSMA PET/CT in prostate cancer patients who have rising PSA After curative treatment and are being considered for targeted therapy. J Nucl Med 2015;56:1185–90.
[107] Afshar-Oromieh A, Avtzi E, Giesel FL, et al. The diagnostic value of PET/CT imaging with the (68)Ga-labelled PSMA ligand HBED-CC in the diagnosis of recurrent prostate cancer. Eur J Nucl Med Mol Imaging 2015;42:197–209.
[108] Eiber M, Maurer T, Souvatzoglou M, et al. Evaluation of hybrid (6)(8)Ga-PSMA Ligand PET/CT in 248 patients with biochemical recurrence after radical prostatectomy. J Nucl Med 2015;56:668–74.
[109] Scher HI, Morris MJ, Stadler WM, et al. Trial design and objectives for castration-resistant prostate cancer: updated recommendations from the Prostate Cancer Clinical Trials Working Group 3. J Clin Oncol 2016;34:1402–18.
[110] Morris MJ, Molina A, Small EJ, et al. Radiographic progression-free survival as a response biomarker in metastatic castration-resistant prostate cancer: COU-AA-302 results. J Clin Oncol 2015;33:1356–63.
[111] Bryce AH, Alumkal JJ, Armstrong A, et al. Radiographic progression with nonrising PSA in metastatic castration-resistant prostate cancer: post hoc analysis of PREVAIL. Prostate Cancer Prostatic Dis 2017;20:221–7.
[112] Evans MJ, Smith-Jones PM, Wongvipat J, et al. Noninvasive measurement of androgen receptor signaling with a positron-emitting radiopharmaceutical that targets prostate-specific membrane antigen. Proc Natl Acad Sci U S A 2011;108:9578–82.
[113] Hope TA, Aggarwal RR, Westphalen AC, Cooperberg MR, Greene KL. Targeted PET imaging for prostate-specific membrane antigen in prostate cancer. Future Oncol 2016;12:2393–6.
[114] Hope TA, Truillet C, Ehman EC, et al. 68Ga-PSMA-11 PET imaging of response to androgen receptor inhibition: first human experience. J Nucl Med 2017;58:81–4.
[115] Wright Jr GL, Grob BM, Haley C, et al. Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. Urology 1996;48:326–34.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

[116] Miyamoto DT, Lee RJ, Stott SL, et al. Androgen receptor signaling in circulating tumor cells as a marker of hormonally responsive prostate cancer. Cancer Discov 2012;2:995–1003.
[117] Meller B, Bremmer F, Sahlmann CO, et al. Alterations in androgen deprivation enhanced prostate-specific membrane antigen (PSMA) expression in prostate cancer cells as a target for diagnostics and therapy. EJNMMI Res 2015;5:66.
[118] Rowe SP, Deville C, Paller C, et al. Uptake of 18F-DCFPyL in Paget's disease of bone, an important potential pitfall in clinical interpretation of PSMA PET studies. Tomography 2015;1:81–4.
[119] Hofman MS, Iravani A. Gallium-68 prostate-specific membrane antigen PET imaging. PET Clin 2017;12:219–34.
[120] De Giorgi U, Caroli P, Scarpi E, et al. (18)F-Fluorocholine PET/CT for early response assessment in patients with metastatic castration-resistant prostate cancer treated with enzalutamide. Eur J Nucl Med Mol Imaging 2015;42:1276–83.
[121] Engert A, Haverkamp H, Kobe C, et al. Reduced-intensity chemotherapy and PET-guided radiotherapy in patients with advanced stage Hodgkin's lymphoma (HD15 trial): a randomised, open-label, phase 3 non-inferiority trial. Lancet 2012;379:1791–9.
[122] Perez-Lopez R, Mateo J, Mossop H, et al. Diffusion-weighted imaging as a treatment response biomarker for evaluating bone metastases in prostate cancer: a pilot study. Radiology 2017;283:168–77.
[123] Fizazi K, Carducci M, Smith M, et al. Denosumab versus zoledronic acid for treatment of bone metastases in men with castration-resistant prostate cancer: a randomised, double-blind study. Lancet 2011;377:813–22.
[124] Saad F. Zoledronic acid significantly reduces pathologic fractures in patients with advanced-stage prostate cancer metastatic to bone. Clin Prostate Cancer 2002;1:145–52.
[125] Saad F, Gleason DM, Murray R, et al. Long-term efficacy of zoledronic acid for the prevention of skeletal complications in patients with metastatic hormone-refractory prostate cancer. J Natl Cancer Inst 2004;96:879–82.
[126] Smith MR, Saad F, Coleman R, et al. Denosumab and bone-metastasis-free survival in men with castration-resistant prostate cancer: results of a phase 3, randomised, placebo-controlled trial. Lancet 2012;379:39–46.
[127] Smith MR, Halabi S, Ryan CJ, et al. Randomized controlled trial of early zoledronic acid in men with castration-sensitive prostate cancer and bone metastases: results of CALGB 90202 (alliance). J Clin Oncol 2014;32:1143–50.
[128] James ND, Pirrie SJ, Pope AM, et al. Clinical outcomes and survival following treatment of metastatic castrate-refractory prostate cancer with docetaxel alone or with strontium-89, zoledronic acid, or both: The TRAPEZE randomized clinical trial. JAMA Oncol 2016;2:493–9.
[129] Smith MR, Coleman RE, Klotz L, et al. Denosumab for the prevention of skeletal complications in metastatic castration-resistant prostate cancer: comparison of skeletal-related events and symptomatic skeletal events. Ann Oncol 2015;26:368–74.
[130] Sartor O, Coleman R, Nilsson S, et al. Effect of radium-223 dichloride on symptomatic skeletal events in patients with castration-resistant prostate cancer and bone metastases: results from a phase 3, double-blind, randomised trial. Lancet Oncol 2014;15:738–46.
[131] Himelstein AL, Foster JC, Khatcheressian JL, et al. Effect of longer-interval vs standard dosing of zoledronic acid on skeletal events in patients with bone metastases: a randomized clinical trial. JAMA 2017;317:48–58.
[132] Patel V, Kelleher M, Sproat C, Kwok J, McGurk M. New cancer therapies and jaw necrosis. Br Dent J 2015;219:203–7.
[133] Saad F, Brown JE, Van Poznak C, et al. Incidence, risk factors, and outcomes of osteonecrosis of the jaw: integrated analysis from three blinded active-controlled phase III trials in cancer patients with bone metastases. Ann Oncol 2012;23:1341–7.
[134] Beltran H, Prandi D, Mosquera JM, et al. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. Nat Med 2016;22:298–305.
[135] Beltran H, Eng K, Mosquera JM, et al. Whole-exome sequencing of metastatic cancer and biomarkers of treatment response. JAMA Oncol 2015;1:466–74.
[136] Aparicio AM, Shen L, Tapia EL, et al. Combined tumor suppressor defects characterize clinically defined aggressive variant prostate cancers. Clin Cancer Res 2016;22:1520–30.
[137] Dardenne E, Beltran H, Benelli M, et al. N-Myc Induces an EZH2-mediated transcriptional program driving neuroendocrine prostate cancer. Cancer Cell 2016;30:563–77.
[138] Bishop JL, Thaper D, Vahid S, et al. The master neural transcription factor BRN2 is an androgen receptor-suppressed driver of neuroendocrine differentiation in prostate cancer. Cancer Discov 2017;7:54–71.
[139] Li Y, Donmez N, Sahinalp C, et al. SRRM4 drives neuroendocrine transdifferentiation of prostate adenocarcinoma under androgen receptor pathway inhibition. Eur Urol 2017;71:68–78.
[140] Mu P, Zhang Z, Benelli M, et al. SOX2 promotes lineage plasticity and antiandrogen resistance in TP53- and RB1-deficient prostate cancer. Science 2017;355:84–8.
[141] Robinson D, Van Allen EM, Wu YM, et al. Integrative clinical genomics of advanced prostate cancer. Cell 2015;161:1215–28.
[142] Mateo J, Carreira S, Sandhu S, et al. DNA-repair defects and olaparib in metastatic prostate cancer. N Engl J Med 2015;373:1697–708.
[143] Ku SY, Rosario S, Wang Y, et al. Rb1 and Trp53 cooperate to suppress prostate cancer lineage plasticity, metastasis, and antiandrogen resistance. Science 2017;355:78–83.
[144] Pritchard CC, Morrissey C, Kumar A, et al. Complex MSH2 and MSH6 mutations in hypermutated microsatellite unstable advanced prostate cancer. Nat Commun 2014 Sep 25;5:4988. http://dx.doi.org/10.1038/ncomms5988.
[145] Wyatt AW, Azad AA, Volik SV, et al. Genomic Alterations in cell-free dna and enzalutamide resistance in castration-resistant prostate cancer. JAMA Oncol 2016;2:1598–606.
[146] Antonarakis ES, Lu C, Wang H, et al. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N Engl J Med 2014;371:1028–38.
[147] Qu F, Xie W, Nakabayashi M, et al. Association of AR-V7 and prostate-specific antigen RNA levels in blood with efficacy of abiraterone acetate and enzalutamide treatment in men with prostate cancer. Clin Cancer Res 2017;23:726–34.
[148] Del Re M, Biasco E, Crucitta S, et al. The detection of androgen receptor splice variant 7 in plasma-derived exosomal RNA strongly predicts resistance to hormonal therapy in metastatic prostate cancer patients. Eur Urol 2017;71:680–7.
[149] Scher HI, Lu D, Schreiber NA, et al. Association of AR-V7 on circulating tumor cells as a treatment-specific biomarker with outcomes and survival in castration-resistant prostate cancer. JAMA Oncol 2016;2:1441–9.
[150] Romanel A, Gasi Tandefelt D, Conteduca V, et al. Plasma AR and abiraterone-resistant prostate cancer. Sci Transl Med 2015;7:312re10.
[151] Grasso CS, Wu YM, Robinson DR, et al. The mutational landscape of lethal castration-resistant prostate cancer. Nature 2012;487:239–43.
[152] Mateo J, Boysen G, Barbieri CE, et al. DNA repair in prostate cancer: biology and clinical implications. Eur Urol 2017;71:417–25.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

[153] Cheng HH, Pritchard CC, Boyd T, Nelson PS, Montgomery B. Biallelic inactivation of BRCA2 in platinum-sensitive metastatic castration-resistant prostate cancer. Eur Urol 2016;69:992–5.
[154] Sternberg CN, Petrylak DP, Sartor O, et al. Multinational, double-blind, phase III study of prednisone and either satraplatin or placebo in patients with castrate-refractory prostate cancer progressing after prior chemotherapy: the SPARC trial. J Clin Oncol 2009;27:5431–8.
[155] Nghiem B, Zhanga X, Lama H-M, et al. Mismatch repair enzyme expression in primary and castrate resistant prostate cancer Asian. J Urol 2016;3:223–8.
[156] Schweizer MT, Cheng HH, Tretiakova MS, et al. Mismatch repair deficiency may be common in ductal adenocarcinoma of the prostate. Oncotarget 2016;7:82504–10.
[157] Le DT, Uram JN, Wang H, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med 2015;372:2509–20.
[158] Lee J, Demissie K, Lu SE, Rhoads GG. Cancer incidence among Korean-American immigrants in the United States and native Koreans in South Korea. Cancer Control 2007;14:78–85.
[159] Mucci LA, Hjelmborg JB, Harris JR, et al. Familial risk and heritability of cancer among twins in Nordic countries. JAMA 2016;315:68–76.
[160] Goldgar DE, Easton DF, Cannon-Albright LA, Skolnick MH. Systematic population-based assessment of cancer risk in first-degree relatives of cancer probands. J Natl Cancer Inst 1994;86:1600–8.
[161] Lange EM. Male Reproductive Cancers: Epidemiology, Pathology and Genetics. New York, NY: Springer; 2010.
[162] Zeigler-Johnson CM, Rennert H, Mittal RD, et al. Evaluation of prostate cancer characteristics in four populations worldwide. Canadian J Urol 2008;15:4056–64.
[163] Cui J, Staples MP, Hopper JL, English DR, McCredie MR, Giles GG. Segregation analyses of 1,476 population-based Australian families affected by prostate cancer. Am J Hum Genet 2001;68:1207–18.
[164] Ahmed M, Eeles R. Germline genetic profiling in prostate cancer: latest developments and potential clinical applications. Future Sci OA 2016;2:FSO87.
[165] Thompson D, Easton D, Breast Cancer Linkage C. Variation in cancer risks, by mutation position, in BRCA2 mutation carriers. Am J Hum Genet 2001;68:410–9.
[166] Kote-Jarai Z, Leongamornlert D, Saunders E, et al. BRCA2 is a moderate penetrance gene contributing to young-onset prostate cancer: implications for genetic testing in prostate cancer patients. Br J Cancer 2011;105:1230–4.
[167] Leongamornlert D, Mahmud N, Tymrakiewicz M, et al. Germline BRCA1 mutations increase prostate cancer risk. Br J Cancer 2012;106:1697–701.
[168] Leongamornlert D, Saunders E, Dadaev T, et al. Frequent germline deleterious mutations in DNA repair genes in familial prostate cancer cases are associated with advanced disease. Br J Cancer 2014;110:1663–72.
[169] Narod SA, Neuhausen S, Vichodez G, et al. Rapid progression of prostate cancer in men with a BRCA2 mutation. Br J Cancer 2008;99:371–4.
[170] Tryggvadottir L, Vidarsdottir L, Thorgeirsson T, et al. Prostate cancer progression and survival in BRCA2 mutation carriers. J Natl Cancer Inst 2007;99:929–35.
[171] Castro E, Goh C, Olmos D, et al. Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer. J Clin Oncol 2013;31:1748–57.
[172] Castro E, Goh C, Leongamornlert D, et al. Effect of BRCA mutations on metastatic relapse and cause-specific survival after radical treatment for localised prostate cancer. Eur Urol 2015;68:186–93.
[173] Pritchard CC, Mateo J, Walsh MF, et al. Inherited DNA-repair gene mutations in men with metastatic prostate cancer. N Engl J Med 2016;375:443–53.
[174] Annala M, Struss WJ, Warner EW, et al. Treatment outcomes and tumor loss of heterozygosity in germline DNA repair-deficient prostate cancer. Eur Urol. In press. http://dx.doi.org/10.1016/j.eururo.2017.02.023.
[175] Na R, Zheng SL, Han M, et al. Germline mutations in ATM and BRCA1/2 distinguish risk for lethal and indolent prostate cancer and are associated with early age at death. Eur Urol 2017;71:740–7.
[176] Bancroft EK, Page EC, Castro E, et al. Targeted prostate cancer screening in BRCA1 and BRCA2 mutation carriers: results from the initial screening round of the IMPACT study. Eur Urol 2014;66:489–99.
[177] Rao K, Manya K, Azad A, et al. Uro-oncology multidisciplinary meetings at an Australian tertiary referral centre–impact on clinical decision-making and implications for patient inclusion. BJU Int 2014;114(Suppl 1):50–4.
[178] Chambers SK, Ferguson M, Gardiner RA, Aitken J, Occhipinti S. Intervening to improve psychological outcomes for men with prostate cancer. Psychooncology 2013;22:1025–34.
[179] Chambers SK, Occhipinti S, Foley E, et al. Mindfulness-based cognitive therapy in advanced prostate cancer: a randomized controlled trial. J Clin Oncol 2017;35:291–7.
[180] Chambers SK, Occhipinti S, Schover L, et al. A randomised controlled trial of a couples-based sexuality intervention for men with localised prostate cancer and their female partners. Psychooncology 2015;24:748–56.
[181] Ussher JM, Perz J, Kellett A, et al. Health-related quality of life, psychological distress, and sexual changes following prostate cancer: a comparison of gay and bisexual men with heterosexual men. J Sex Med 2016;13:425–34.
[182] Friedenreich CM, Wang Q, Neilson HK, Kopciuk KA, McGregor SE, Courneya KS. Physical activity and survival after prostate cancer. Eur Urol 2016;70:576–85.
[183] Galvao DA, Nosaka K, Taaffe DR, et al. Resistance training and reduction of treatment side effects in prostate cancer patients. Med Sci Sports Exerc 2006;38:2045–52.
[184] Galvao DA, Spry N, Denham J, et al. A multicentre year-long randomised controlled trial of exercise training targeting physical functioning in men with prostate cancer previously treated with androgen suppression and radiation from TROG 03.04 RADAR. Eur Urol 2014;65:856–64.
[185] Droz JP, Albrand G, Gillessen S, et al. Management of prostate cancer in elderly patients: recommendations of a Task Force of the International Society of Geriatric Oncology. Eur Urol. In press. http://dx.doi.org/10.1016/j.eururo.2016.12.025.
[186] Kalsi T, Babic-Illman G, Ross PJ, et al. The impact of comprehensive geriatric assessment interventions on tolerance to chemotherapy in older people. Br J Cancer 2015;112:1435–44.
[187] Keating NL, O'Malley A, Freedland SJ, Smith MR. Diabetes and cardiovascular disease during androgen deprivation therapy: observational study of veterans with prostate cancer. J Natl Cancer Inst 2012;104:1518–23.
[188] Keating NL, O'Malley AJ, Freedland SJ, Smith MR. Diabetes and cardiovascular disease during androgen deprivation therapy: observational study of veterans with prostate cancer. J Natl Cancer Inst 2010;102:39–46.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

[189] Keating NL, O'Malley AJ, Smith MR. Diabetes and cardiovascular disease during androgen deprivation therapy for prostate cancer. J Clin Oncol 2006;24:4448–56.
[190] Alibhai SM, Duong-Hua M, Sutradhar R, et al. Impact of androgen deprivation therapy on cardiovascular disease and diabetes. J Clin Oncol 2009;27:3452–8.
[191] Smith MR, Finkelstein JS, McGovern FJ, et al. Changes in body composition during androgen deprivation therapy for prostate cancer. J Clin Endocrinol Metab 2002;87:599–603.
[192] Smith MR, Lee H, Nathan DM. Insulin sensitivity during combined androgen blockade for prostate cancer. J Clin Endocrinol Metab 2006;91:1305–8.
[193] Nead KT, Gaskin G, Chester C, et al. Androgen deprivation therapy and future Alzheimer's disease risk. J Clin Oncol 2016;34:566–71.
[194] Nead KT, Gaskin G, Chester C, Swisher-McClure S, Leeper NJ, Shah NH. Association between androgen deprivation therapy and risk of dementia. JAMA Oncol 2017;3:49–55.
[195] Khosrow-Khavar F, Rej S, Yin H, Aprikian A, Azoulay L. Androgen deprivation therapy and the risk of dementia in patients with prostate cancer. J Clin Oncol 2017;35:201–7.
[196] Alibhai SM, Breunis H, Timilshina N, et al. Impact of androgen-deprivation therapy on cognitive function in men with nonmetastatic prostate cancer. J Clin Oncol 2010;28:5030–7.

Please cite this article in press as: Gillessen S, et al. Management of Patients with Advanced Prostate Cancer: The Report of the Advanced Prostate Cancer Consensus Conference APCCC 2017. Eur Urol (2017), http://dx.doi.org/10.1016/j.eururo.2017.06.002

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

MSH2 Loss in Primary Prostate Cancer

Liana B. Guedes[1]*, Emmanuel S. Antonarakis[2]*, Michael T. Schweizer[3], Nooshin Mirkheshti[1], Fawaz Almutairi[1], Jong Chul Park[2], Stephanie Glavaris[1], Jessica Hicks[1], Mario A. Eisenberger[2], Angelo M. De Marzo[1,2,4], Jonathan I. Epstein[1,2,4], William B. Isaacs[4], James R. Eshleman[1,2], Colin C. Pritchard[5]**, Tamara L. Lotan[1,2]**

Departments of [1]Pathology and [2]Oncology, Johns Hopkins University School of Medicine; [3]Department of Medicine, Division of Oncology, University of Washington, Seattle, WA; [4]Brady Urological Institute, Johns Hopkins University School of Medicine; [5]Department of Laboratory Medicine, University of Washington, Seattle, WA.

*Equal contribution
**Equal contribution

Running Title: MSH2 Loss in Primary Prostate Cancer

Key Words: Prostatic adenocarcinoma; MSH2; mismatch repair, microsatellite instability; hypermutation.

To whom correspondence should be addressed:
Tamara Lotan, MD
1550 Orleans Street
Baltimore, MD 21231
(410) 614-9196 (ph)
e-mail: tlotan1@jhmi.edu

Financial Support: Funding for this research was provided in part by a Transformative Impact Award from the CDMRP (W81XWH-12-PCRP-TIA, TLL), the Prostate Cancer Foundation (CCP), and by PCRP award PC131820 (CCP). Additional funding and resources were provided by the NIH/NCI Prostate SPORE P50CA58236, the NIH/NCI PNW Prostate SPORE CA097186 and NCI Cancer Center Support Grant 5P30 CA015704-40.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Abstract:

Purpose: Inactivation of mismatch repair (MMR) genes may predict sensitivity to immunotherapy in metastatic prostate cancers. We studied primary prostate tumors with MMR defects.

Experimental Design: 1133 primary prostatic adenocarcinomas and 43 prostatic small cell carcinomas (NEPC) were screened by MSH2 immunohistochemistry with confirmation by next-generation sequencing (NGS). Microsatellite instability (MSI) was assessed by PCR and NGS (mSINGS).

Results: Of primary adenocarcinomas and NEPC, 1.2% (14/1176) had MSH2 loss. Overall, 8% (7/91) of adenocarcinomas with primary Gleason pattern 5 (Gleason score 9-10) had MSH2 loss compared to 0.4% (5/1042) of tumors with any other scores ($p<0.05$). 5% (2/43) of NEPC had MSH2 loss. MSH2 was generally homogenously lost, suggesting it was an early/clonal event. NGS confirmed *MSH2* loss-of-function alterations in all (12/12) samples, with bi-allelic inactivation in 83% (10/12) and hypermutation in 83% (10/12). Overall, 61% (8/13) and 58% (7/12) of patients had definite MSI by PCR and mSINGS, respectively. Three patients (25%) had germline mutations in *MSH2*. Tumors with MSH2 loss had a higher density of infiltrating CD8+ lymphocytes compared to grade-matched controls without MSH2 loss (390 vs. 76 cells/mm^2; $p=0.008$), and CD8+ density was correlated with mutation burden among cases with MSH2 loss ($r=0.72$, $p=0.005$). T-cell receptor sequencing on a subset revealed a trend towards higher clonality in cases versus controls.

Conclusion: Loss of MSH2 protein is correlated with *MSH2* inactivation, hypermutation and higher tumor-infiltrating lymphocyte density, and appears most common among very high-grade primary tumors, where routine screening may be warranted if validated in additional cohorts.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Translational Relevance: Inactivation of mismatch repair (MMR) genes is associated with microsatellite instability (MSI) and hypermutation in metastatic prostate cancers and may predict response to immunotherapy. To screen for MMR defects in primary prostate cancers, where alterations are rare and standard DNA sequencing may miss complex rearrangements, we used an immunohistochemistry (IHC) assay for MSH2. We find that MSH2 loss is enriched among primary tumors with high-grade histology, is an early and clonal event, and is highly predictive of underlying *MSH2* genomic alteration, hypermutation and high CD8+ lymphocyte density. In contrast to observations in colorectal carcinoma, only about half of primary prostate tumors with *MSH2* inactivation have evidence of MSI by PCR and/or next-generation sequencing assays using traditional cutoffs. These data have implications for the testing of primary tumor specimens for MMR defects in the setting of metastatic prostate cancer for which pembrolizumab may be a treatment option following recent FDA approval.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Introduction:

Approximately 10% of advanced/metastatic prostate tumors have a markedly elevated rate of single nucleotide mutations (1, 2), almost always due to underlying somatic and/or germline inactivation of genes in the mismatch repair (MMR) family (*MSH2*, *MSH6*, *MLH1* or *PMS2*) and often accompanied by microsatellite instability (MSI) (1), similar to what has been observed in colorectal carcinoma (3). Similarly, a significant fraction of the commonly used prostate cancer cell lines have bi-allelic loss of MMR genes, including DU145 (4, 5), LNCaP (5-7), CWR22RV1 (8), and VCaP cells (8). Taken together, this work in advanced tumors and cell lines suggests that the rate of MMR defects in prostate cancers may be similar to the prevalence seen in colorectal carcinoma (~15% of cases). Importantly, advanced prostate tumors with MMR gene loss and hypermutation may respond favorably to immunotherapies targeted to PD-1 (9, 10) and/or CTLA-4, similar to what has been seen in colorectal carcinoma, due to the generation of neoepitopes and resulting immune recognition of “non-self” tumor antigens (11, 12).

Though previous studies have focused on MMR defects in advanced prostate cancer, the relative frequency and clinical significance of MMR alterations in primary prostate cancer is less certain. Most studies describing the prevalence of microsatellite instability in primary prostate cancer were performed more than a decade ago and a wide range of MSI frequency (2 to 65%) has been reported (13-15). The numbers and types of microsatellite markers used to define MSI in these older studies differed significantly from international

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

standardized guidelines subsequently developed for MSI testing in colorectal carcinomas (16, 17). When current MSI definitions are super-imposed on these earlier studies, the MSI prevalence in prostate cancers is rarely higher than 10% overall (18). Indeed, more recent work using the previously recommended mono- and di-nucleotide marker panels from the Bethesda Consensus Panel (16, 17) has suggested that the rate of MSI in primary prostate tumors is <4% (19) similar to recent genomic profiling studies of primary prostate cancer where the rate of MMR gene loss was even lower, <3% (20). Even rarer, recent studies of Lynch syndrome, an autosomal dominant condition associated with increased incidence of early colorectal and endometrial carcinomas due to germline MMR gene inactivation, have suggested that increased risk of prostate carcinoma is likely part of the syndrome (21-28), though not all studies are consistent (29, 30). Small series of Lynch-associated prostate cancer patients have found that some, though notably not all, prostate tumors arising in this setting are associated with MSI and there may be an association with increased tumor-infiltrating lymphocytes and higher pathologic grade (21, 26).

Given the relative rarity of MSI and MMR gene alterations in primary prostate cancers, few studies have characterized primary prostate tumors with MMR gene inactivation outside of Lynch syndrome. This is of particular interest and clinical relevance with the recent FDA-approval of the PD-1 inhibitor pembrolizumab to treat metastatic tumors of all histologic types with MMR deficiency or MSI. To identify and molecularly characterize primary prostate tumors with sporadic and/or germline MMR defects, we utilized an

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

immunohistochemistry (IHC) assay for MSH2. We initially focused on MSH2 because this MMR protein was the most robustly expressed in primary prostate tumors, is the most commonly altered MMR gene in advanced prostate cancer (1, 20), and the MMR gene most frequently implicated in Lynch syndrome patients who develop microsatellite-unstable prostate cancer (21-26). Screening for MSH2 loss by IHC is particularly useful in the setting of primary prostate cancer, since it can be easily applied to large numbers of tumors and large tumor areas to screen for the relatively rare tumors with protein loss. In addition, it is potentially more sensitive than standard whole-exome or targeted sequencing protocols, which may miss the complex genomic rearrangements that commonly involve MMR genes in prostate cancer (1). Herein, we pathologically and molecularly characterize primary prostate tumors with MSH2 protein loss.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Materials and Methods:

Patients and tissue samples: In accordance with the US Common Rule and after institutional review board (IRB) approval, a total of 8 partially overlapping tissue microarray (TMA) cohorts containing a total of 1290 (n=1133 unique) samples of prostatic adenocarcinomas from radical prostatectomies performed at Johns Hopkins were queried using MSH2 immunohistochemistry. Most of these cohorts have been previously described, and notably many were created to enrich for adverse oncologic outcomes, so they do not represent an unbiased survey of a radical prostatectomy population. In brief, these consisted of: 1) A cohort of consecutive tumors at radical prostatectomy from 2000-2004, including all tumors with Gleason score >6 (n=462 samples) (31); 2) A cohort of high-grade (Gleason score 9/10) tumors at radical prostatectomy from 1998-2005, designed for comparison to high-grade urothelial carcinomas (n=28) (32); 3) A cohort of all radical prostatectomies from 2004-2014 with primary Gleason pattern 5 and available clinical follow-up (n=71); 4) A cohort of African-American radical prostatectomy samples from 2005-2010, all with Gleason score 4+3=7 and higher (n=84) (31); 5) A cohort of patients who all developed metastatic disease and were treated with abiraterone/enzalutamide after radical prostatectomy at Johns Hopkins from 1995-2011 (n=34); 6) A cohort of patients with ductal adenocarcinoma and/or cribriform Gleason score 8 adenocarcinoma at radical prostatectomy from 1984-2004 (n=46) (33); 7) A case-cohort study of men undergoing radical prostatectomy from 1992-2009 who subsequently developed metastatic disease (n=325) (34); and 8) A cohort of men with biochemical

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

recurrence following radical prostatectomy from 1992-2009 (n=240) (35). 9) Finally, a separate cohort of 43 neuroendocrine prostate carcinomas (NEPC) with confirmed small cell carcinoma histology on TMA was also queried by MSH2 IHC (36). Additional control tissues were procured from a radical prostatectomy sample from a patient with a known pathogenic germline mutation in *MSH2*, as well as from an additional 10 prostatectomy specimens with tumors with primary Gleason pattern 5 but intact MSH2 immunostaining.

Finally, electropherograms from an additional 10 cases of colorectal carcinoma that were MSI-H by PCR and tested within the last year were utilized to compare differences in microsatellite marker shifts between prostate and colorectal carcinoma with MMR defects.

Cell line TMA: 56 cell lines from the NCI-60 cell line panel (Developmental Therapeutics Program, NCI) were used to evaluate MSH2 IHC staining. All cell lines were pelleted, fixed in 10% neutral buffered formalin and processed and cut as tissue. Cell lines were punched and tissue microarrays created as previously described (37). STR genotyping was completed once prior to creation of the cell line TMA.

Mismatch repair protein immunohistochemistry and interpretation: MMR protein IHC was performed on the Ventana Benchmark autostaining system utilizing

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

primary antibodies from Ventana (Roche/Ventana Medical Systems, Tucson, AZ). MSH2 IHC used a mouse monoclonal antibody (clone G219-1129), MSH6 IHC used a mouse monoclonal antibody (clone 44), MLH1 IHC used a mouse monoclonal antibody (clone M1) and PMS2 IHC used a rabbit monoclonal antibody (clone EPR3947). All samples were incubated with primary antibody after antigen retrieval in CC1 buffer, and primary antibody incubation was followed by detection with the UltraView HRP system (Roche/Ventana Medical Systems, Tucson, AZ). Each tissue microarray spot or standard histologic section containing tumor cells was visually dichotomously scored for presence or absence of cytoplasmic MMR protein signal by a urologic pathologist blinded to the sequencing/MSI testing data (TLL). A spot was considered to show MMR protein loss if any tumor cells in any tumor spot showed MMR protein loss, with intact staining in admixed benign prostate glands and/or surrounding stromal cells, endothelial cells or lymphocytes. Spots without internal control staining were considered ambiguous and not scored. All samples were initially screened for MSH2 loss by scoring TMA spots; however for all cases with MSH2 loss on TMA, confirmatory immunostaining for MSH2, MSH6, MLH1 and PMS2 was also performed on standard histologic tissue sections.

DNA isolation: For samples from the TMAs, a total of five 0.6 μm punches were procured from the same tumor and benign areas in the paraffin block sampled on the TMA. For standard histologic sections, tumor and normal tissue was macrodissected guided by hematoxylin and eosin stained section. DNA was

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

extracted from FFPE material using the Qiagen FFPE DNA extraction kit (Hilden, Germany) according to the manufacturer's directions. DNA concentrations were quantified with the Qubit fluorometer, using a Quant-iT dsDNA High Sensitivity Assay Kit (Invitrogen, Carlsbad, CA).

PCR-based microsatellite instability analysis: MSI analyses were carried out using multiplex PCR with fluorescently-labelled primers, included in the MSI Analysis System, Version 1.2 (Promega Corp. Madison, WI, USA), for amplification of five mononucleotide repeat markers (NR-21, BAT-26, BAT-25, NR-24, MONO-27) and two pentanucleotide repeat loci (Penta-C and Penta-D) to confirm identity between the tumor and benign tissue pair. The PCR reactions were performed in samples containing at least 250 ng of DNA, 0.05U/µl TaqGold (Applied Biosystems) and sterile dH_2O (Sigma). The PCR was performed using a Veriti Thermal Cycler (ThermoFisher Scientific) using the following program: 95°C 11min, 96°C for 1min, 10 cycles of 94°C for 30s, 58°C for 30s, 70°C for 1min; 20 cycles of 90°C for 30s, 58°C for 30s, 70°C for 1min; and 60°C for 30min. PCR products were mixed with formamide and size standard, denatured and run on an ABI (Waltham, MA) 3130 capillary electrophoresis instrument using injection times of 30-180 seconds. Cancers were designated MSI-H with 2 shifts, MSI-L with 1 shift and MSS with no shifts relative to the germline pattern. The pattern and number of bases shifted were compared to the first 10 MSI-H colorectal cancers diagnosed in 2016. Bimodal and trimodal patterns consisted

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

of one or two additional (non-germline peaks), where the novel peak was distinct in that bases in between it and the germline peak had lower fluorescent intensity. Shoulder pattern had an extension of peaks (bases of equal or lower intensity) beyond those that could be attributed to germline peaks injected for different times. In some cases, we observed the presence of single base shifts in peaks of one of the markers without any further changes in other markers, and these cases were not classified as unstable.

Targeted Next-generation sequencing and MSI by NGS (mSINGS): Targeted next-generation deep sequencing of MMR genes and MSI by NGS (mSINGS) analysis was performed using UW-OncoPlex (http://web.labmed.washington.edu/tests/genetics/UW-OncoPlex) as previously described (38, 39). UW-OncoPlex is a clinically-validated assay performed in the CLIA-laboratory setting that sequences to 500x average depth all exons, introns, and flanking regions of *MSH2*, *MSH6*, and *MLH1* and all exons of *PMS2* and *EPCAM*. Genomic libraries were made from 1µg of genomic DNA extracted from prostate tumor and matched normal (germline) formalin-fixed paraffin embedded tissue and a custom Agilent SureSelect XT capture set used for target enrichment. After target enrichment and barcoding, libraries were pooled and sequenced on an Illumina NextSeq 500 instrument with paired-end 101bp reads. A custom bioinformatics pipeline detects single nucleotide variants, indels of all sizes, structural rearrangements, *PMS2* pseudogene disambiguation, and copy number changes. mSINGS analysis was performed on UW-OncoPlex data as

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

previously described using a total of 65 mononucleotide microsatellite loci (40). Total mutation burden was estimated from targeted sequencing data as previously described with a threshold of 12 coding mutations/Mb for hypermutation (39, 41). Sequencing interpretation was done by an expert molecular pathologist (CCP) who was blinded to clinical data and other molecular testing results.

CD3, CD8 and PD-L1 immunostaining and digital image quantification: CD8 and CD3 immunostaining was performed on standard histologic slides in a CLIA-accredited laboratory using a mouse monoclonal antibody for CD8 (clone C8/C8144B, 760-4250; Cell Marque, Rocklin, CA) and rabbit polyclonal antibody for CD3 (A0452, Dako/Agilent Technologies, Santa Clara, CA) with antigen detection by the Ventana iView system (Roche/Ventana Medical Systems, Tucson, AZ). PD-L1 immunostaining was performed using a rabbit monoclonal antibody (SP142, Ventana) on the Ventana Benchmark platform, also using standard histologic sections. For image analysis of CD8 immunostaining, a single standard histologic slide stained with CD8 was scanned at 20x magnification on the Aperio Scanscope AT Turbo (Leica, Wetzlar, Germany). CD8+ and CD3+ cells per millimeter squared tissue was quantitatively performed with the Aperio Digital Pathology software (Leica, Wetzlar, Germany). For each immunostained standard slide, all tumor tissue present, excluding benign epithelium, with minimal intervening stromal tissue was selected for analysis. An average of

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

67 mm^2 of tumor tissue area was selected for analysis (range: 16-152 mm^2). CD8+ or CD3+ cells within the selected tumor area were identified by Aperio software as previously described (42), and the ratio of CD8+ or CD3+ cells to the total tumor area analyzed was calculated for each case. PD-L1 staining was scored as positive if >1% of immune cells or tumor cells showed PD-L1 membranous positivity.

T-cell receptor sequencing (TCR-seq): TCR-seq of the CDR3 variable region of the T-cell receptor β chain was performed as described previously (43) on a subset of 6 samples (3 cases with MSH2 loss and 3 primary Gleason pattern 5 controls) (Adaptive Biotechnologies, Seattle, WA). Briefly 2-3 µg of DNA was prepared as described above from tumor samples using macrodissection of standard histologic sections. Once prepared, DNA was transferred to Adaptive Technologies for sequencing. TCR metrics and clonality indices were calculated using the ImmunoSeq Analyzer (44).

Statistical Methods:

Statistical analysis was performed using Student's t-test, Fisher's exact test and linear regression. P-values of <0.05 were considered statistically significant.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Results:

Initial validation of MSH2 immunohistochemistry using prostate cancer cell lines and tumor tissues with known *MSH2*–mutant genomic status: For initial validation, we performed MSH2 immunohistochemistry (IHC) on prostate cancer cell lines with and without known alterations in MSH2 (**Supplementary Figure S1**). DU145 cells have a heterozygous splice site mutation in *MLH1* with missense mutation in the other genes (4, 5) and had intact staining for MSH2 and MSH6, with loss of MLH1 and PMS2 as expected. PC3 cells have intact MMR genes by sequencing (8) and by immunohistochemistry. LNCaP cells have a homozygous deletion of *MSH2* and *MSH6* (5-7), and showed loss of MSH2 and MSH6 immunostaining. VCaP cells have a heterozygous frameshift mutation in *MSH6* (c.1085) (8) and showed intact MSH2 immunostaining. Finally, CWR22RV1 cells have a homozygous deletion of *MSH2* and *MSH6* (8) and showed loss of MSH2 and MSH6 staining, with intact staining for MLH1 and PMS2. To begin to assess the assay in primary prostate tumor samples, we utilized a radical prostatectomy sample from a patient with a known germline pathogenic mutation in *MSH2* (p.A636P, (45, 46)) and somatic loss of heterozygosity (i.e. confirmed bi-allelic inactivation), which was MSI-high (MSI-H) by PCR (3/5 markers shifted) and MSI-positive by mSINGS (though notably without evidence of clear-cut hypermutation, at only 9 mutations/Mb, possibly due to low tumor DNA content). In this sample, MSH2 and MSH6 protein expression was entirely absent by IHC (**Supplementary Figure S1**).

Clinical-pathologic features of cases with MSH2 loss by immunohistochemistry (IHC): Next, we screened for MSH2 protein loss in tissue microarray spots from a total of 1176

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

unique primary prostate carcinomas, including 1133 prostatic adenocarcinomas and 43 prostatic small cell neuroendocrine carcinomas (NEPC). Altogether, 1.2% (14/1176) of prostate primaries had MSH2 loss, including 1% (12/1133) of primary adenocarcinomas and 5% (2/43) of NEPC cases (**Figures 1** and **2**). Clinical-pathologic characteristics of these cases are detailed in **Table 1** and the Gleason grade distribution of the adenocarcinomas queried by MSH2 immunostaining is detailed in **Supplementary Table S1**. The average patient age of cases with MSH2 loss was 62 years, which was not significantly different from the overall cohort of 1176 cases (59 years; p=0.53). Tumors with MSH2 loss were generally extremely aggressive by pathologic features, including tumor grade (**Supplementary Figure S2**) and stage. Overall, 71% (10/14) of the cases with MSH2 loss were either Gleason score 9 adenocarcinomas or NEPC cases. The four remaining cases included one case of Gleason score 8, and three with Gleason score 7 (see **Table 1** for breakdown), though one had tertiary Gleason pattern 5 cancer. Of the 12 adenocarcinoma cases at radical prostatectomy which had pathologic stage information available, 50% (6/12) were pathologic stage pT3b or higher (two with nodal involvement), 33% (4/12) were pT3a and 17% (2/12) were pT2. When adenocarcinomas were analyzed separately, 8% (7/91) of tumors with primary Gleason pattern 5 (5+4=9 or 5+5=10) cases had MSH2 loss compared to less than 1% of tumors with all other grades (p<0.0001). Interestingly, there seemed to be a much greater enrichment for MSH2 loss among primary Gleason pattern 5 cases, even when compared to Gleason score 4+5=9 cases (7/91 vs 1/108; p=0.02).

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

For cases with MSH2 loss by TMA screening, confirmation of loss was performed on standard histologic sections, along with immunostaining for MSH6, MLH1 and PMS2. All cases with MSH2 loss showed concordant MSH6 loss, as expected since stability of the proteins is only ensured as heterodimers, with intact MLH1 and PMS2 (**Supplementary Figure S1**, **Supplementary Figure S3**). Notably, staining for MSH6 in stromal cells was often quite weak and focal, making it difficult to use this stain to screen large numbers of cases for MSH6 loss in tumor cells (**Supplementary Figure S3**). When all cases were evaluated on standard histologic slides, MSH2 staining was homogenously lost in all tumor cells sampled in the dominant tumor nodule from each case, suggesting that it was an early and clonal event in the evolution of the tumor. This is in stark contrast to other genomic alterations that we have profiled *in situ*, such as *PTEN* deletion (47).

MSH2 sequencing: To confirm that our immunoassay was detecting underlying genomic alterations at the *MSH2* locus, we analyzed normal and tumor DNA from all cases with MSH2 loss using a targeted next-generation sequencing (NGS) assay specifically designed to detect somatic/germline mutations as well as small and large-scale genomic rearrangements at the MMR gene loci (38). We did not sequence unselected (i.e. MSH2-intact) cases in this study since nearly 500 cases of primary prostate cancer have been sequenced to date in the TCGA effort, with excellent representation of Gleason score 9 tumors (20). In these studies, the median mutation burden has been less than 1 mutation/Mb of coding DNA, regardless of tumor grade, with only 1% of unselected primary tumors showing genomic alterations in *MSH2*. In our cases with

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

MSH2 loss, NGS confirmed (at least mono-allelic) *MSH2* loss-of-function alterations in all (12/12) samples with adequate tumor DNA available for analysis. Two cases did not have enough DNA for sequencing. Definite evidence of bi-allelic inactivation was present in 83% (10/12) of cases, with the 2 cases that lacked evidence of bi-allelic deletion both showing low tumor content, which can make loss-of-heterozygosity calls challenging from sequencing data; one of these two cases showed possible LOH. Cases without apparent bi-allelic inactivation were indistinguishable from cases with two-copy loss of *MSH2* based on MSH2 and MSH6 immunostaining (**Supplementary Figure S4**), suggesting that loss of the second copy was likely present but not detected by sequencing. Somatic and germline alterations are described in **Table 2**. Overall, 25% (3/12) of cases showed somatic large-scale deletions and/or genomic rearrangements involving both the *MSH2* and *MSH6* loci, including one case with a deletion involving *MSH2* exons 3-16 and all of MSH6, one case with *MSH2* bi-allelic copy loss and another case with a large-scale rearrangement involving both loci, including a 5.7 Mb inversion (**Figure 1**). All of these cases demonstrated loss of heterozygosity. The remaining cases showed predominantly small deletions resulting in frameshift or splice site alterations in *MSH2*, with a rare missense mutation known to affect splicing (p.G669V) (**Figure 1**). Overall, 25% (3/12) of cases showed germline pathogenic lesions in *MSH2*, including a frameshift, a splice site and a nonsense mutation (**Figure 1**). Two of these cases had somatic loss of heterozygosity or other somatic inactivation consistent with a second hit to the gene in the tumor DNA only, and another case had likely loss of heterozygosity. Only one of the three patients with germline *MSH2* inactivation had a documented history of Lynch syndrome with a prior

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

colorectal carcinoma and upper tract urothelial carcinoma. Two other patients had no known history of Lynch syndrome, though one had a prior colorectal carcinoma and both had a strong family history of colorectal and other Lynch-associated carcinomas.

PCR-based microsatellite instability: Thirteen of the fourteen cases with MSH2 protein loss had interpretable MSI testing by PCR; one case failed MSI testing in several replicates, likely due to the presence of PCR inhibitors. Overall, only 61% (8/13) of these had evidence of MSI by PCR, though analysis was frequently limited by low overall DNA amplification level (48, 49). Of those classified as unstable by the PCR assay, 7/8 had 2 or more microsatellite markers with signs of instability (MSI-H) and 1/8 had only one shifted marker (MSI-L), though this case had low amplification.

Among the prostate cases with evidence of microsatellite instability there were discrete bimodal peak shifts of 2-6 bases (mean 4 bases) and a high prevalence of shoulder pattern shifts (13/21 or 62% of unstable loci had a shoulder pattern with remaining unstable loci showing a bimodal pattern) (**Figure 3**, **Supplementary Table S2**). These findings were notably more subtle than those seen in 10 colorectal cancer controls with MSI-H, where peak shifts of 4-13 bases were observed (mean 7 bases), with a predominance of bimodal and trimodal shifts in all peaks (only 3/47 or 6% of unstable loci showed a shoulder pattern, with 72% showing a bimodal pattern and 21% showing a trimodal pattern). Among prostate cases, there was no apparent predominance of shifts in one marker over the other. There was notable failure of amplification of the BAT-26 marker in most (11/14) samples, possibly due to the presence of amplification inhibitors in the FFPE-extracted DNA. The presence of 4

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

amplified markers is still sufficient to make MSI calls if there is presence of 2 or more markers demonstrating MSI (16). In one case, however, there was one shifted marker (BAT-25) among 3 amplified ones, this case was considered MSI-L.

mSINGS: Twelve of the fourteen cases with MSH2 protein loss had adequate DNA available for sequencing. Overall, only 58% (7/12) cases had definite evidence of MSI by mSINGS at a cutoff of >20% unstable loci (38), though analysis was frequently limited by low tumor content in the analyzed DNA, which must be above 20% for this validated assay (**Table 2, Supplementary Table S3**). Among the 5 cases that did not have definitive MSI by mSINGS, three were indeterminate (one of which had inadequate tumor purity), and two were negative (both of which had inadequate tumor content). Cases that were MSI-H by PCR were likely to be MSI by mSINGS. Of the cases that were MSI-H by PCR assay with sequencing data, 67% (4/6) were positive for MSI by mSINGS, with one case that was negative by mSINGS but with inadequate tumor purity, and one case slightly below threshold for calling MSI by mSINGS (15% of loci queries, scored as indeterminate). Interestingly, cases that were MSS by PCR were also likely to be positive or indeterminate for MSI by mSINGS. Of the cases that were MSS by PCR assay, 40% (2/5) were positive for MSI by mSINGS and 40% (2/5) were indeterminate by mSINGS with evidence of MSI at 18% and 15% of loci queried. The remaining microsatellite stable (MSS) case by PCR was negative for MSI by mSINGS, but showed low tumor content.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Mutation burden: Hypermutation, defined as more than 12 mutations per Mb on the 1.3 Mb NGS panel, was present in 83% (10/12) of tumors with MSH2 loss by immunostaining, and cases had a median of 26 (range: 3-104) mutations/Mb. The case with the highest mutation burden (104 mutations/Mb, considered to be ultramutated) had an additional somatic mutation in *POLD1* involving the exonuclease "proofreading" domain (p.D402N) which likely contributed to the ultra-high mutation burden. The patient with the lowest mutation burden (3 mutations/Mb) was also negative for MSI by mSINGS and was MSS by MSI-PCR, though the mSINGS result was limited by low tumor content.

Infiltrating lymphocyte quantification and TCR-seq: Tumor infiltrating lymphocytes (TIL) appeared increased by hematoxylin and eosin staining in many cases with MSH2 protein loss, though there was notable variability (**Supplementary Figure S2**). By immunostaining, we digitally quantified the number of CD3+ (**Figure 4A**) and CD8+ TIL (**Figure 4B**) in each case with MSH2 loss and 10 primary Gleason pattern 5 cases without MSH2 protein loss using a single standard histologic section of tumor for each radical prostatectomy. CD3+ and CD8+ lymphocyte density (quantified on adjacent tissue sections) were highly correlated across cases (r=0.94) and controls (r=0.84), thus we focused on the CD8+ fraction in further analysis (**Figure 4B**). There was a mean of 390 CD8+ cells/mm^2 among the cases with MSH2 loss, significantly higher than the mean of 76 CD8+ cells/mm^2 seen among the 10 grade-matched control cases (p=0.008, **Figure 4C**). Similarly, the CD8 to CD3 cell ratio was significantly higher among cases (mean=0.59) compared to controls (mean=0.29, p<0.001), which together

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

with the increased absolute number of CD8+ cells, suggests a more prominent cytotoxic lymphocytic response among the tumors with MSH2 loss compared to those without. Clinical-pathologic variables or the presence of an underlying germline alteration in *MSH2* did not correlate appreciably with the number of CD3 or CD8 positive cells/mm^2, as some cases with germline alterations had very high lymphocyte counts and some had quite low counts. Similarly, the presence of bi-allelic MSH2 inactivation and MSI status of the tumor by either PCR or sequencing did not show obvious association with lymphocyte count. Strikingly, however, the quantitative CD8+ lymphocyte density was significantly correlated with the overall mutation burden among the 12 cases with MSH2 loss and available sequencing data (r=0.7235, p=0.005, Spearman's correlation coefficient, **Figure 4D**). PD-L1 staining (defined as the presence of >1% positive cells among immune cells or tumor cells) was positive in 50% (7/14) of tumors with MSH2 loss, however positivity was most commonly seen in the immune cell compartment (**Supplementary Figure S5**). PD-L1 positive cases tended to have higher lymphocyte counts (and mutation burden) overall (**Figure 4D**), with one notable exception seen in an NEPC case with low lymphocyte counts where sequencing data was not available. TCR-seq was performed on a small subset of 3 cases and 3 controls with adequate DNA and relatively lower tissue block age per recommendations that blocks less than 5-10 years of age be utilized for this assay (**Supplementary Table S4**). As expected given the differences in lymphocyte counts, the mean number of templates available for sequencing was higher in the cases compared to the controls (10590 vs 4628). There was a trend towards a higher mean productive clonality (0.079 vs 0.042) in cases compared to controls, though this did not reach statistical significance in this small

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

sample size. Notably, there was marked variation among the cases with MSH2 loss in terms of productive clonality indices (0.043 to 0.117) that was not obviously correlated with any other genomic or lymphocyte metrics.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Discussion:

The findings in the current study support the concept that MSH2 protein loss, as measured by immunohistochemistry (IHC), is highly correlated with underlying genomic inactivation of *MSH2* and hypermutation. Our study is among the first to compare contemporary MSH2 IHC to next-generation sequencing in primary prostate tumors (9), and the first to do so in a large number of specimens. Use of this IHC assay enabled us to screen >1100 primary tumors to identify the relatively rare cases with MMR defects, comprising only about 1% of cases our cohorts. Accordingly, this is among the first studies to examine the phenotype of sporadic primary prostate tumors with MMR defects. Perhaps the most interesting phenotypic correlation discovered here is that MSH2 loss appears more common among very-high-grade prostatic primary tumors, with rates approaching 10% among tumors with primary Gleason pattern 5 in our series. These data are particularly striking since we only queried one of four genes known to be involved in MMR, suggesting that the true rate of MMR gene alterations in this population is very likely to be even higher. Clearly, given the small cohort examined, additional validation studies are required to confirm this association. However, these findings are generally consistent with previous reports of high-grade prostate cancer in Lynch syndrome patients, particularly among those with microsatellite instability (21, 26, 50). If validated in subsequent studies, these data argue for routine clinical screening of very-high-risk patients for germline and sporadic MMR gene loss using IHC or other techniques.

The high Gleason grade of most tumors with MSH2 loss, combined with the overall enrichment of MMR defects among metastatic compared to primary cases,

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

suggests that these tumors may behave aggressively from the outset, in contrast to what has been observed in MMR defective colorectal cancers. Many of the prostate tumors with MSH2 loss in our study had significantly increased CD8+ lymphocyte density. The presence of a marked lymphocytic infiltrate, which is also frequently seen in colorectal tumors with MMR loss, may contribute to the undifferentiated, high-grade appearance of the tumor in some cases (51). This phenomenon is also commonly seen in lymphoepithelioma-like carcinomas (52) and medullary tumors of the breast (53), which are not associated with MMR defects and in all of these cases, the presence of high-grade carcinoma may not always be well-correlated with aggressive tumor progression. However, beyond the appearance of high histologic grade, the potentially aggressive behavior of primary prostate tumors with MSH2 loss was also supported by their generally high pathological stage in the current series. It may also be consistent with the relatively higher rate of MMR defects among advanced or metastatic prostate cancer cases (1, 2) compared to primary tumors (20), as well as the enrichment of MMR defects observed in aggressive variants of prostate cancer, including ductal adenocarcinoma (9) and potentially NEPC. Unfortunately, we had insufficient clinical follow-up data and biased selection of tumors for screening in the current study, both of which precluded comparison of long-term oncologic outcomes among cases with MSH2 loss and those with intact MMR. This will be the focus of future studies.

Our use of an *in situ* assay to examine MSH2 status led to the observation that MSH2 protein loss is almost always homogeneous within a given tumor nodule. This is notable, given the fact that only a minority of our cases had germline alterations in *MSH2*, and suggests that bi-allelic somatic inactivation of MSH2 is frequently an early

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

clonal event when it occurs. This is in stark contrast to other common genomic alterations in primary prostate cancer, such as *PTEN* deletion or *TP53* mutation which are also enriched in metastatic and castration-resistant disease (47, 54, 55) and manifest a much more heterogeneous staining pattern in the primary tumor. Though we did select for cases with more homogeneous alterations in MSH2 by screening for loss using tissue microarray (TMA) punches, PTEN heterogeneity may be easily captured in TMA punches (47, 54), suggesting that this was not likely a major confounder.

In our cohort with MSH2 protein and genomic loss, the MSI PCR assay was substantially less sensitive for MSH2 loss than has been previously described for other tumor types. MSI PCR testing is generally ~95% sensitive for underlying genomic alteration in *MSH2* in colorectal carcinoma meta-analyses (56). Rare discordant cases generally show intact IHC, with evidence of MSI by PCR, often due to functionally deleterious missense mutations that fail to compromise protein expression. However, cases of colorectal carcinoma with clear genomic loss by DNA sequencing but absence of microsatellite instability by PCR are extraordinarily rare to our knowledge. Similarly, high concordance of MSI PCR and MSH2 immunohistochemistry has also been observed in endometrial carcinomas (57). In contrast, among our prostatic primaries, only 61% (8/13) of cases with MSH2 protein loss had evidence of MSI by PCR, including one case which was unstable at only one microsatellite, consistent with MSI-L status. The low sensitivity of traditional MSI markers in primary prostate carcinoma is paralleled by the more subtle peak shifts observed in prostate tumors in our study, compared to those typically seen in colorectal carcinoma. Though studies in colorectal carcinoma are abundant (56), few contemporary studies have compared MMR IHC

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

assays or genomic testing to MSI PCR results in primary prostate cancer outside of the context of Lynch syndrome, and older studies have shown only weak correlations (58). In a more recent study of Lynch syndrome patients, only 66% (4/6) of prostatic adenocarcinomas with MSH2/6 protein loss showed evidence of MSI by PCR-based testing, however, this study did not use the contemporary Promega 5 marker microsatellite panel (17, 48, 50). In a second study, 88% (7/8) of Lynch prostatic carcinomas with MSH2/6 protein loss showed evidence of MSI by PCR-based testing, though it is notable that 5/7 of the cases with MSI were categorized as MSI-L, meaning only one of five markers was unstable (26). Similar to our results in the current study, these data suggest that contemporary PCR panels may be inadequate to screen for MMR defects in primary prostate cancer.

There is emerging evidence that MSI testing by next-generation sequencing is at least as, and potentially more, sensitive for MSI than traditional PCR-based testing (40). MSI testing by sequencing interrogates a much larger panel of microsatellite loci than PCR testing, which could increase sensitivity. In addition, the 5 mononucleotide repeat markers that make up the standard MSI PCR testing panel were largely designed for detection of MSI in colorectal carcinoma, and perhaps are not optimized for similar studies in prostate carcinoma where alternative microsatellites may be more sensitive markers of MSI. However, using previously established cutoffs of 20% of unstable loci to call MSI, mSINGS did not have a markedly different sensitivity for cases with MSH2 protein loss than PCR testing (58% vs 61%) in our study; however, these data are limited by the low tumor content (below the 20% cutoff) in 25% of our samples (including the only samples that were entirely negative for MSI by mSINGS). In

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

addition, we had a number of indeterminate cases, with MSI at some loci but not reaching the 20% threshold, such that decreasing the threshold to 15% of tested loci with instability was sufficient to raise the sensitivity to 83%. Further optimization of NGS MSI assays are needed, and should ideally be performed on samples with a high tumor content.

Regardless, both the mSINGS data and MSI-PCR results seem to point to a similar conclusion that MSI in primary prostate cancer is likely more subtle and difficult to detect compared to that seen in colorectal cancer. The reasons for this difference remain unclear. It is possible that prostate samples have relatively lower tumor content compared to colorectal tumor samples, which can decrease the sensitivity of MSI testing by both methods. It is also tempting to speculate that the relatively low proliferation and apoptosis rates in primary prostate cancer may be one contributing factor. Since MSI increases over time with errors accrued after each cell division, and the absolute proliferation rate in primary prostate cancer is generally lower than that in colorectal cancer, tumors from the prostate (even if of equal size to those in the colon) may have undergone markedly fewer cell divisions, contributing to the lower level of MSI in these prostate tumors. Consistent with this hypothesis, MSI PCR assays were much more concordant with underlying MMR gene genomic status in advanced metastatic prostate cancer than we found in our primary tumors (1), perhaps suggesting that more extended genomic evolution is required for manifestation of the MSI phenotype.

In this context, hypermutation may be a more sensitive marker of underlying *MSH2* genomic loss than MSI testing in our cohort, since hypermutation was present in

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

all but two of the cases with MSH2 loss (83%). There were some cases with hypermutation in the absence of MSI, suggesting that hypermutation may precede, or perhaps occur in the absence of, microsatellite instability in primary prostate cancer, and that this might be the more sensitive marker of underlying MMR defects in primary prostate cancer. Remarkably, the mutation burden in tumors with MSH2 loss was highly correlated with infiltrating lymphocyte density, a finding that potentially corroborates the anecdotal response of these tumors to immunotherapy (9, 10). Overall, both the absolute number of CD8+ lymphocytes was increased among tumors with MSH2 loss, as well as the relative proportion of the CD3+ cells that were cytotoxic T cells (the CD8/CD3 ratio). Though the prognostic significance of this ratio is unclear, these data are consistent with a more prominent cytotoxic T-cell response among the MSH2-null tumors in our cohort. However, more detailed additional immunophenotypic studies are required to definitively test this. Importantly, however, there was a wide variation in both mutation burden and the lymphocytic response among prostate primaries with MSH2 loss, and this variability was not easily explained by underlying genomic alteration in *MSH2.* Future studies will examine whether mutation burden and/or lymphocyte density or clonality index by TCR-seq are predictive biomarkers for duration of response to immune checkpoint blockade in the prostate and other organs.

Our study has some important limitations. First, we focused on only a single MMR protein, MSH2, for validation. This was in large part because protein expression of MSH6, MLH1 and PMS2 appeared to be considerably weaker than MSH2 expression in the prostate using IHC assays validated for colorectal carcinoma (see MSH6 in Supplementary Figure S3); we are currently working to further optimize these assays for

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

screening similar to what we did with MSH2. In addition, loss of MSH2 is most common in prostate cancer compared to MSH6, MLH1 and PMS2 (1, 2, 20). However, this single assay will clearly lack sensitivity for screening prostate tumors for MMR defects as it will miss alterations in the other MMR genes. Due to the design of our study, we also cannot give an accurate estimate of the true prevalence of MSH2 loss in unselected primary prostate cancers. Though we screened >1100 primary tumors for loss, many of these cases were selected for inclusion on TMAs designed to enrich for adverse oncologic outcomes, which may confound our prevalence estimates. Future studies in high-risk populations where sequencing is performed on all tumors screened by IHC will be useful to address prevalence and IHC assay sensitivity questions.

Collectively, our data have important implications for screening algorithms used to identify prostate cancer patients that may benefit from immune checkpoint blockade. Although it remains debated, our cases add additional evidence that prostate cancer is, definitively, a Lynch syndrome-associated tumor. Our study suggests that MMR gene alterations are commonly clonal and homogenous in primary prostate tumors, which should facilitate screening of primary tumor samples (even those collected on needle biopsies) for MSH2 deficiency, and suggests that heterogeneity between metastases is likely to be rare (although differences in MSI and hypermutation status are possible). In addition, we demonstrate that, pending validation in independent cohorts, the highest rates of MSH2 loss are among tumors with the most aggressive pathologic features, namely primary Gleason pattern 5 and neuroendocrine prostate carcinomas. Given the generally poor oncologic outcomes in these groups, these data suggest that screening this population routinely for MMR defects may be useful, perhaps even at diagnosis, to

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

potentially direct patients towards immunotherapy. The relatively subtle MSI by PCR assays in many primary prostate tumors with genomic *MSH2* loss is intriguing and indicates that MSI PCR using the contemporary markers developed for colorectal carcinoma may be an inadequate test in isolation for primary prostate carcinomas. Indeed, screening by next-generation sequencing for hypermutation may be among the most sensitive genomic tests in this context and since tumor infiltrating CD8+ cell density is highly correlated with mutation burden, this may also provide an additional screening tool for labs that do not have ready access to sequencing. Finally, assessing for MMR protein loss by IHC remains an excellent and relatively inexpensive test to screen for underlying genomic alterations in MMR genes, especially if future studies can optimize and validate MSH6, MLH1 and PMS2 IHC assays. Ultimately, these IHC assays may be paired with mutation burden analysis for routine screening of high-risk populations and to stratify patients for clinical trials of immune checkpoint blockade therapy.

Acknowledgements: The authors would like to thank Chrisley Pickens, Rachel Mercado and Emily Adams for outstanding technical assistance with the MSI PCR testing and Mallory Beightol for assistance with DNA sequencing.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

References:

1. Pritchard CC, Morrissey C, Kumar A, Zhang X, Smith C, Coleman I, et al. Complex MSH2 and MSH6 mutations in hypermutated microsatellite unstable advanced prostate cancer. Nat Commun. 2014;5:4988.
2. Robinson D, Van Allen EM, Wu YM, Schultz N, Lonigro RJ, Mosquera JM, et al. Integrative clinical genomics of advanced prostate cancer. Cell. 2015;161(5):1215-28.
3. Eshleman JR, Lang EZ, Bowerfind GK, Parsons R, Vogelstein B, Willson JK, et al. Increased mutation rate at the hprt locus accompanies microsatellite instability in colon cancer. Oncogene. 1995;10(1):33-7.
4. Boyer JC, Umar A, Risinger JI, Lipford JR, Kane M, Yin S, et al. Microsatellite instability, mismatch repair deficiency, and genetic defects in human cancer cell lines. Cancer Res. 1995;55(24):6063-70.
5. Chen Y, Wang J, Fraig MM, Metcalf J, Turner WR, Bissada NK, et al. Defects of DNA mismatch repair in human prostate cancer. Cancer Res. 2001;61(10):4112-21.
6. Leach FS, Velasco A, Hsieh JT, Sagalowsky AI, McConnell JD. The mismatch repair gene hMSH2 is mutated in the prostate cancer cell line LNCaP. J Urol. 2000;164(5):1830-3.
7. Yeh CC, Lee C, Dahiya R. DNA mismatch repair enzyme activity and gene expression in prostate cancer. Biochem Biophys Res Commun. 2001;285(2):409-13.
8. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature. 2012;483(7391):603-7.
9. Schweizer MT, Cheng HH, Tretiakova MS, Vakar-Lopez F, Klemfuss N, Konnick EQ, et al. Mismatch repair deficiency may be common in ductal adenocarcinoma of the prostate. Oncotarget. 2016;7(50):82504-10.
10. Graff JN, Alumkal JJ, Drake CG, Thomas GV, Redmond WL, Farhad M, et al. Early evidence of anti-PD-1 activity in enzalutamide-resistant prostate cancer. Oncotarget. 2016;7(33):52810-7.
11. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med. 2015;372(26):2509-20.
12. Lee V, Murphy A, Le DT, Diaz LA, Jr. Mismatch Repair Deficiency and Response to Immune Checkpoint Blockade. The oncologist. 2016;21(10):1200-11.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

13. Gao X, Wu N, Grignon D, Zacharek A, Liu H, Salkowski A, et al. High frequency of mutator phenotype in human prostatic adenocarcinoma. Oncogene. 1994;9(10):2999-3003.
14. Uchida T, Wada C, Wang C, Ishida H, Egawa S, Yokoyama E, et al. Microsatellite instability in prostate cancer. Oncogene. 1995;10(5):1019-22.
15. Egawa S, Uchida T, Suyama K, Wang C, Ohori M, Irie S, et al. Genomic instability of microsatellite repeats in prostate cancer: relationship to clinicopathological variables. Cancer Res. 1995;55(11):2418-21.
16. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 1998;58(22):5248-57.
17. Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Ruschoff J, et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst. 2004;96(4):261-8.
18. Leach FS. Microsatellite instability and prostate cancer: clinical and pathological implications. Current opinion in urology. 2002;12(5):407-11.
19. Azzouzi AR, Catto JW, Rehman I, Larre S, Roupret M, Feeley KM, et al. Clinically localised prostate cancer is microsatellite stable. BJU Int. 2007;99(5):1031-5.
20. Cancer Genome Atlas Research N. The Molecular Taxonomy of Primary Prostate Cancer. Cell. 2015;163(4):1011-25.
21. Bauer CM, Ray AM, Halstead-Nussloch BA, Dekker RG, Raymond VM, Gruber SB, et al. Hereditary prostate cancer as a feature of Lynch syndrome. Familial cancer. 2011;10(1):37-42.
22. Raymond VM, Mukherjee B, Wang F, Huang SC, Stoffel EM, Kastrinos F, et al. Elevated risk of prostate cancer among men with Lynch syndrome. J Clin Oncol. 2013;31(14):1713-8.
23. Win AK. Is prostate cancer a Lynch syndrome cancer? Asian journal of andrology. 2013;15(5):588-9.
24. Ryan S, Jenkins MA, Win AK. Risk of prostate cancer in Lynch syndrome: a systematic review and meta-analysis. Cancer Epidemiol Biomarkers Prev. 2014;23(3):437-49.
25. Haraldsdottir S, Hampel H, Wei L, Wu C, Frankel W, Bekaii-Saab T, et al. Prostate cancer incidence in males with Lynch syndrome. Genetics in medicine : official journal of the American College of Medical Genetics. 2014;16(7):553-7.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

26. Dominguez-Valentin M, Joost P, Therkildsen C, Jonsson M, Rambech E, Nilbert M. Frequent mismatch-repair defects link prostate cancer to Lynch syndrome. BMC urology. 2016;16:15.
27. Soravia C, van der Klift H, Brundler MA, Blouin JL, Wijnen J, Hutter P, et al. Prostate cancer is part of the hereditary non-polyposis colorectal cancer (HNPCC) tumor spectrum. American journal of medical genetics Part A. 2003;121A(2):159-62.
28. Grindedal EM, Moller P, Eeles R, Stormorken AT, Bowitz-Lothe IM, Landro SM, et al. Germ-line mutations in mismatch repair genes associated with prostate cancer. Cancer Epidemiol Biomarkers Prev. 2009;18(9):2460-7.
29. Dowty JG, Win AK, Buchanan DD, Lindor NM, Macrae FA, Clendenning M, et al. Cancer risks for MLH1 and MSH2 mutation carriers. Human mutation. 2013;34(3):490-7.
30. Aarnio M, Sankila R, Pukkala E, Salovaara R, Aaltonen LA, de la Chapelle A, et al. Cancer risk in mutation carriers of DNA-mismatch-repair genes. International journal of cancer. 1999;81(2):214-8.
31. Tosoian JJ, Almutairi F, Morais CL, Glavaris S, Hicks J, Sundi D, et al. Prevalence and Prognostic Significance of PTEN Loss in African-American and European-American Men Undergoing Radical Prostatectomy. Eur Urol. 2016.
32. Chuang AY, DeMarzo AM, Veltri RW, Sharma RB, Bieberich CJ, Epstein JI. Immunohistochemical differentiation of high-grade prostate carcinoma from urothelial carcinoma. The American Journal of Surgical Pathology. 2007;31(8):1246-55.
33. Herawi M, Epstein JI. Immunohistochemical antibody cocktail staining (p63/HMWCK/AMACR) of ductal adenocarcinoma and Gleason pattern 4 cribriform and noncribriform acinar adenocarcinomas of the prostate. Am J Surg Pathol. 2007;31(6):889-94.
34. Ross AE, Johnson MH, Yousefi K, Davicioni E, Netto GJ, Marchionni L, et al. Tissue-based Genomics Augments Post-prostatectomy Risk Stratification in a Natural History Cohort of Intermediate- and High-Risk Men. Eur Urol. 2016;69(1):157-65.
35. Johnson MH, Ross AE, Alshalalfa M, Erho N, Yousefi K, Glavaris S, et al. SPINK1 Defines a Molecular Subtype of Prostate Cancer in Men with More Rapid Progression in an at Risk, Natural History Radical Prostatectomy Cohort. J Urol. 2016.
36. Tan HL, Sood A, Rahimi HA, Wang W, Gupta N, Hicks J, et al. Rb loss is characteristic of prostatic small cell neuroendocrine carcinoma. Clin Cancer Res. 2014;20(4):890-903.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

37. Lotan TL, Gurel B, Sutcliffe S, Esopi D, Liu W, Xu J, et al. PTEN protein loss by immunostaining: analytic validation and prognostic indicator for a high risk surgical cohort of prostate cancer patients. Clin Cancer Res. 2011;17(20):6563-73.
38. Pritchard CC, Salipante SJ, Koehler K, Smith C, Scroggins S, Wood B, et al. Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. J Mol Diagn. 2014;16(1):56-67.
39. Cohen SA, Turner EH, Beightol MB, Jacobson A, Gooley TA, Salipante SJ, et al. Frequent PIK3CA Mutations in Colorectal and Endometrial Tumors With 2 or More Somatic Mutations in Mismatch Repair Genes. Gastroenterology. 2016;151(3):440-7 e1.
40. Salipante SJ, Scroggins SM, Hampel HL, Turner EH, Pritchard CC. Microsatellite instability detection by next generation sequencing. Clinical chemistry. 2014;60(9):1192-9.
41. Haraldsdottir S, Hampel H, Tomsic J, Frankel WL, Pearlman R, de la Chapelle A, et al. Colon and endometrial cancers with mismatch repair deficiency can arise from somatic, rather than germline, mutations. Gastroenterology. 2014;147(6):1308-16 e1.
42. Guedes LB, Morais CL, Almutairi F, Haffner MC, Zheng Q, Isaacs JT, et al. Analytic Validation of RNA In Situ Hybridization (RISH) for AR and AR-V7 Expression in Human Prostate Cancer. Clin Cancer Res. 2016;22(18):4651-63.
43. Robins HS, Campregher PV, Srivastava SK, Wacher A, Turtle CJ, Kahsai O, et al. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. Blood. 2009;114(19):4099-107.
44. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature. 2014;515(7528):568-71.
45. Foulkes WD, Thiffault I, Gruber SB, Horwitz M, Hamel N, Lee C, et al. The founder mutation MSH2*1906G-->C is an important cause of hereditary nonpolyposis colorectal cancer in the Ashkenazi Jewish population. American journal of human genetics. 2002;71(6):1395-412.
46. Guillem JG, Rapaport BS, Kirchhoff T, Kolachana P, Nafa K, Glogowski E, et al. A636P is associated with early-onset colon cancer in Ashkenazi Jews. J Am Coll Surg. 2003;196(2):222-5.
47. Ahearn TU, Pettersson A, Ebot EM, Gerke T, Graff RE, Morais CL, et al. A Prospective Investigation of PTEN Loss and ERG Expression in Lethal Prostate Cancer. J Natl Cancer Inst. 2016;108(2).

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

48. Bacher JW, Flanagan LA, Smalley RL, Nassif NA, Burgart LJ, Halberg RB, et al. Development of a fluorescent multiplex assay for detection of MSI-High tumors. Dis Markers. 2004;20(4-5):237-50.
49. Murphy KM, Zhang S, Geiger T, Hafez MJ, Bacher J, Berg KD, et al. Comparison of the microsatellite instability analysis system and the Bethesda panel for the determination of microsatellite instability in colorectal cancers. J Mol Diagn. 2006;8(3):305-11.
50. Rosty C, Walsh MD, Lindor NM, Thibodeau SN, Mundt E, Gallinger S, et al. High prevalence of mismatch repair deficiency in prostate cancers diagnosed in mismatch repair gene mutation carriers from the colon cancer family registry. Familial cancer. 2014;13(4):573-82.
51. Alexander J, Watanabe T, Wu TT, Rashid A, Li S, Hamilton SR. Histopathological identification of colon cancer with microsatellite instability. Am J Pathol. 2001;158(2):527-35.
52. Amin MB, Ro JY, Lee KM, Ordonez NG, Dinney CP, Gulley ML, et al. Lymphoepithelioma-like carcinoma of the urinary bladder. Am J Surg Pathol. 1994;18(5):466-73.
53. Rapin V, Contesso G, Mouriesse H, Bertin F, Lacombe MJ, Piekarski JD, et al. Medullary breast carcinoma. A reevaluation of 95 cases of breast cancer with inflammatory stroma. Cancer. 1988;61(12):2503-10.
54. Lotan TL, Wei W, Morais CL, Hawley ST, Fazli L, Hurtado-Coll A, et al. PTEN Loss as Determined by Clinical-grade Immunohistochemistry Assay Is Associated with Worse Recurrence-free Survival in Prostate Cancer. Eur Urol Focus. 2016;2(2):180-8.
55. Hong MK, Macintyre G, Wedge DC, Van Loo P, Patel K, Lunke S, et al. Tracking the origins and drivers of subclonal metastatic expansion in prostate cancer. Nat Commun. 2015;6:6605.
56. Shia J. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part I. The utility of immunohistochemistry. J Mol Diagn. 2008;10(4):293-300.
57. Modica I, Soslow RA, Black D, Tornos C, Kauff N, Shia J. Utility of immunohistochemistry in predicting microsatellite instability in endometrial carcinoma. Am J Surg Pathol. 2007;31(5):744-51.
58. Velasco A, Hewitt SM, Albert PS, Hossein M, Rosenberg H, Martinez C, et al. Differential expression of the mismatch repair gene hMSH2 in malignant prostate tissue is associated with cancer recurrence. Cancer. 2002;94(3):690-9.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Table 1: Clinical-pathologic characteristics of primary prostate tumors with MSH2 loss by immunohistochemistry

BlockID	TMA	Specimen Type	Tissue type	Year	Age	Race	Gland Weight	Gleason Primary	Gleason Secondary	Gleason Sum	Path Stage	Known Lynch syndrome?
58319	1	RP	AdCa	2001	48	W	38	3	4	7 (tertiary 5)	T3AN0	Yes
66254	3	RP	AdCa	2009	63	W	52.4	5	4	9	T3BN0	No
19236	6	RP	AdCa	2001	64	W	52	4	4	8	T3AN0	No
55795	1	RP	AdCa	2003	63	W	56	4	5	9	T2N0	No
34128	1	RP	AdCa	2001	65	W	40	4	3	7	T3BN0	No
55836	3	RP	AdCa	2005	65	W	69.6	5	4	9	T2N0	No
71503	3	RP	AdCa	2011	69	W	43.7	5	4	9	T3AN0	No
61879	3	RP	AdCa	2014	58	W	56.4	5	4	9	T3BN0	No
35566	9	TURP	NEPC	2001	72			NA	NA	NA		No
22966	2	RP	AdCa	2005	63	W	84.8	5	4	9	T3BN1	No
71484	3	RP	AdCa	2005	66	W	35	5	4	9	T3AN0	No
35592/3	9	TURP	NEPC	2007	79			NA	NA	NA		No
60913	3	RP	AdCa	2010	47	H	66	5	4	9	T3BN1	No
3131	7	RP	AdCa	1993	56	W	54.8	3	4	7	T3BN0	No
22533	control	RP	AdCa	1993	55	W	60.4	5	4	9	T3BN0	No

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Table 2: Molecular characteristics of primary prostate tumors with MSH2 loss by immunohistochemistry. (*=tumor content <20%, ¥=low amplification; NA= not assessed; **coding only out of 1.3 Mb)

ID	Somatic MMR Alteration(s)	LOH	Germline MMR status	Known Lynch syndrome?	MSI-PCR	MS markers shifted	MSI (mSINGS)	Hyper-mutation	Total Mutation Burden**	Mutations/ Mb Coding	Other Mutations found	CD8/mm^2	PD-L1 +
58319	LOH	yes	MSH2 c.892C>T (p.Q298*)	Yes	MSS	0 of 4	IND (15%)	no	13	10	CHEK2 (p.W93Gfs*17); EPHB6 (p.L881Cfs*39); NF2 (p.R336Q); FANCA (exon 3-6del?)	145	no
66254	MSH2 c.2235_2237del (p.I747del)	yes	none	No	MSI-H	2 of 4	IND (15%)	yes	45	34	PBRM1 (p.R58*); ARID1A (p.R1223C, p.K1072Nfs*21); TP53 (p.R306*)	535	yes
19236	MSH2 c.1728del (p.I577Lfs*13)	no*	none	No	MSS	0 of 5	NEG*	no*	3	3	SPOP (p.F102V)	83	no
55795	MSH2 c.1613del (p.N538Tfs*5) + c.547C>T (p.Q183*)	no*	none	No	MSI-H	2 of 4	NEG*	yes*	36	28	AR (p.R727H)	85	no
34128	MSH2 c.1276+2T>A (splicing)	yes	none	No	MSI-H	2 of 4	POS	yes	16	13	MSH6 (p.F1088Lfs*5); ARAF (p.R103W); GNAS (p.R81M); CDK8 (p.R356*)	350	yes
55836	MSH2/6 locus rearrangement (5.7Mb inversion)	yes	none	No	fail	NA	POS	yes	31	24	MSH6 (exon 3-6del?); FOXA1 (p.H247Y, p.M59I); ARID1A (p.R1074W, p.R1733Q)	633	no
71503	MSH2 c.830del (p.L277*) + c.2201C>A (p.S734Y)	no	MSH2 c.1226_1227del (p.Q409Rfs*7)	No	MSS	0 of 5	POS	Yes (ultra)	138	104	MSH6 (p.N534Efs*4); POLD1 (p.D402N)	1016	yes
61879	MSH2 exon 3-16 MSH6 del	yes	none	No	MSI-H	3 of 4	POS	yes	101	76	PIK3CA (p.E726K, p.H1047R, p.E81K)	523	yes
35566	MSH2 bi-allelic copy loss	yes	NA	No	MSS	0 of 4	POS	yes	26	20	RB1 (p.R73Sfs*36); TP53 (p.R175H); RB1 (p.R73Sfs*36)	22	NA
22966	none	possible	MSH2 c.942+3 A>T (splice site mutation)	No	MSI-H	4 of 4	POS	yes	59	45	PTEN (p.R173C, p.R130Q); TP53 (p.R342*)	1020	yes
71484	MSH2 c.2006G>T (p.G669V) + c.943-10T>A (splicing)	no*	none	No	MSS¥	0 of 4	IND (18%)*	yes*	31	24	none	331	no
35592/3	ND	ND	ND	No	MSI-H	2 of 4	ND	ND	ND	ND	ND	114	yes
60913	MSH2 c.646-2A>G (splicing)	yes	none	No	MSI-H	2 of 4	POS	yes	35	27	CSF1R (p.W839*, p.W839*); PIK3CA (p.H1047R); PTEN (p.R233*)	527	yes
3131	ND	ND	ND	No	MSI-L¥	1 of 3	ND	ND	ND	ND	ND	70	no
22533 (control)	LOH	yes	MSH2 c.1906G>C (p.A636P)	No	MSI-H	3 of 5	POS	no*	11	9	TMPRSS2 (p.A347Lfs*5)	72	ND

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure Legends:

FIGURE 1: Representative MSH2 immunostaining in formalin fixed and paraffin embedded primary prostate tumors with biallelic *MSH2* inactivation. Top row: Gleason score 5+4=9 prostate tumors with intact nuclear immunostaining and wild type *MSH2* gene. Second and third rows: tumors with loss of MSH2 expression and somatic two copy *MSH2* genomic inactivation. Although in some sections a weakly positive cytoplasmic stain of unknown significance can be observed, the nuclei remain negative in all tumor cells, with intact staining in stromal cells, lymphocytes and benign epithelium in all cases as an internal positive control. Bottom row: Representative MSH2 immunostaining in formalin fixed and paraffin embedded primary prostate tumors with germline and somatic *MSH2* gene inactivation. Both tumors lack nuclear staining for MSH2. Adjacent benign prostatic glands and stromal cells maintain nuclear expression of MSH2 as an internal control. All photomicrographs are reduced from 200x.

FIGURE 2: Representative MSH2 immunostaining in formalin fixed and paraffin embedded small cell neuroendocrine carcinoma (NEPC) of the prostate. Standard histologic tissue sections of a small cell carcinoma (35595) shows robust MSH2 nuclear staining while two other small cell carcinoma tumors (35592 and 35566) lacking nuclear staining with intact stromal and lymphocyte staining. All photomicrographs are reduced from 200x.

FIGURE 3: **Representative electropherograms of colorectal carcinoma and prostatic adenocarcinoma cases that are MSI-H.** MSI-PCR testing (Promega panel) for representative colorectal carcinoma and primary prostate carcinoma samples. Colorectal tumor sample shows a clear bi-modal pattern with distinct peak shifts in NR-21, BAT-25, MONO-27 mononucleotide markers (new peaks present in tumor sample but absent in normal sample are indicated by vertical arrows). In contrast, the MSI prostate tumor sample shows a bi-modal shift in NR-21 of only six bases (indicated by vertical arrow) and a subtle shift of MONO-27 (“shoulder” morphology, indicated by horizontal arrow).

FIGURE 4: CD8+ tumor infiltrating lymphocyte density in primary prostate tumors with MSH2 loss. (A) Immunostaining for CD3 identifies a high number of tumor infiltrating lymphocytes in a prostate tumor with MSH2 loss, case 71503 (upper panel). Aperio image analysis software is useful to identify CD3+ cells (red) in selected tumor regions and surrounding tumor and stromal nuclei (blue) (lower panel). **(B)** Immunostaining for CD8 identifies a high number of tumor infiltrating lymphocytes in a prostate tumor with MSH2 loss, case 71503 (upper panel). Aperio image analysis software is useful to identify CD8+ cells (red) in selected tumor regions and surrounding nuclei (blue) (lower panel). CD8 and CD3 cell counts were highly correlated in all cases with MSH2 loss and controls without MSH2 loss. **(C)** Mean density of CD8+ infiltrating

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

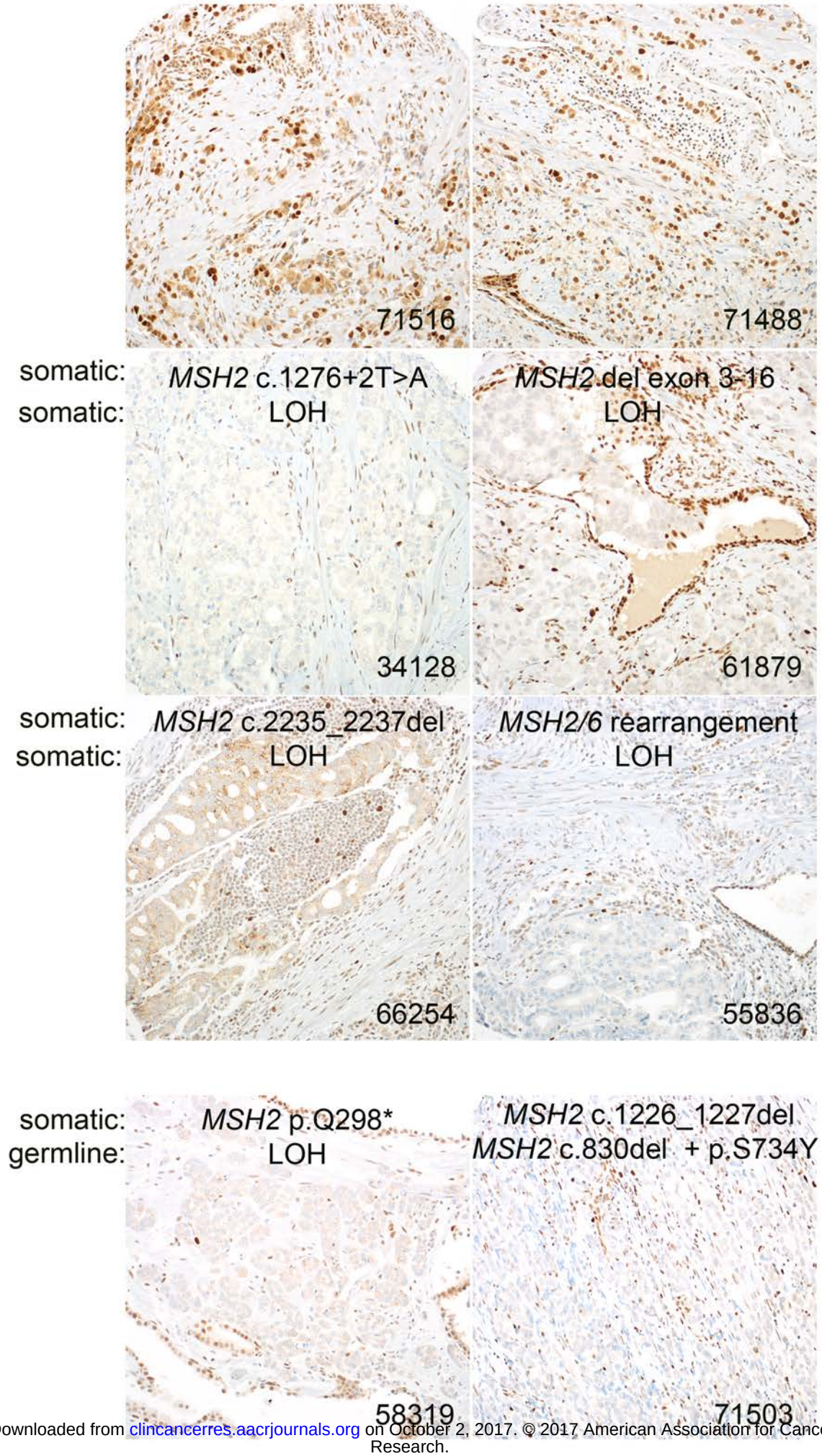
Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

lymphocytes are significantly higher in cases with MSH2 loss compared to matched control tumors with MSH2 intact and primary Gleason pattern 5. **(D)** Density of CD8+ infiltrating lymphocytes is significantly correlated with mutation burden among tumors with MSH2 loss.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

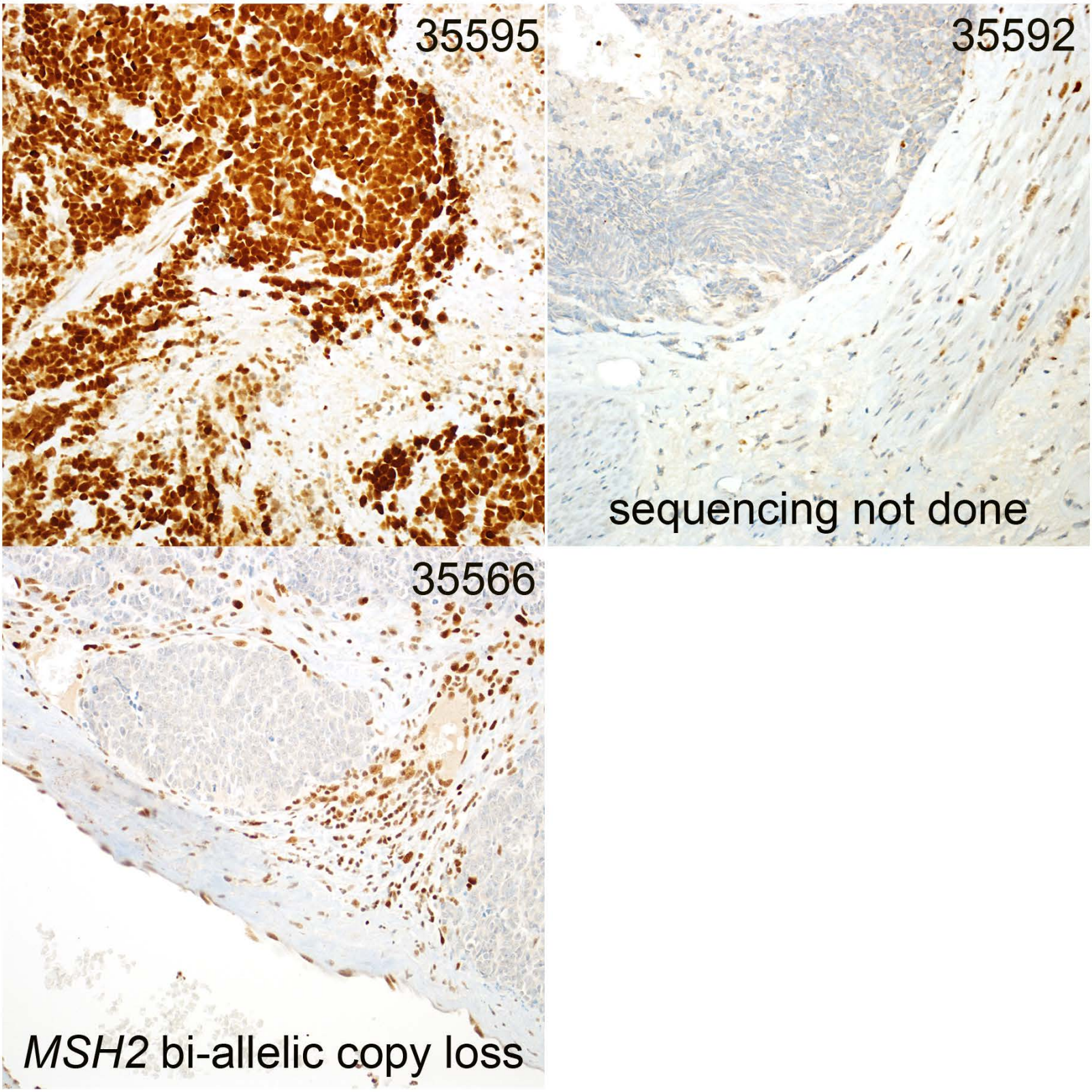
Figure 1



Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

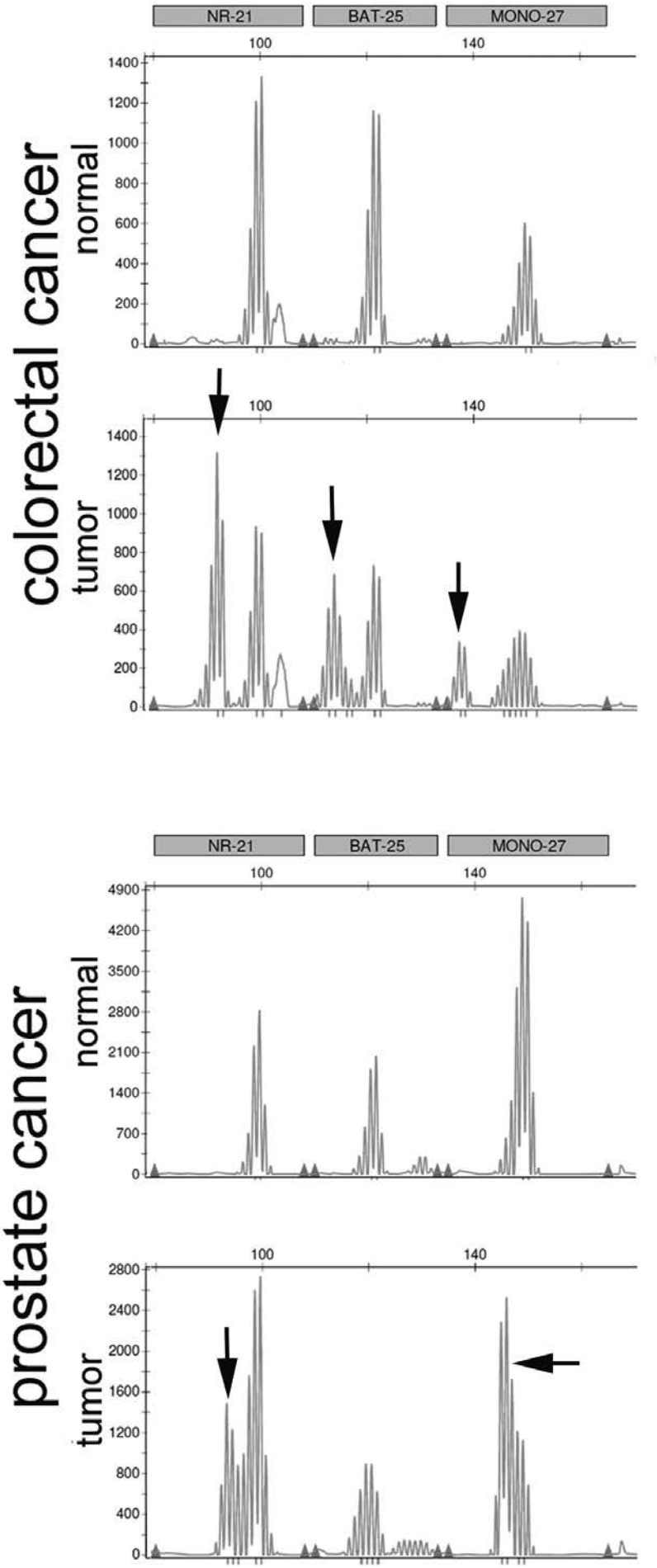
Figure 2



Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

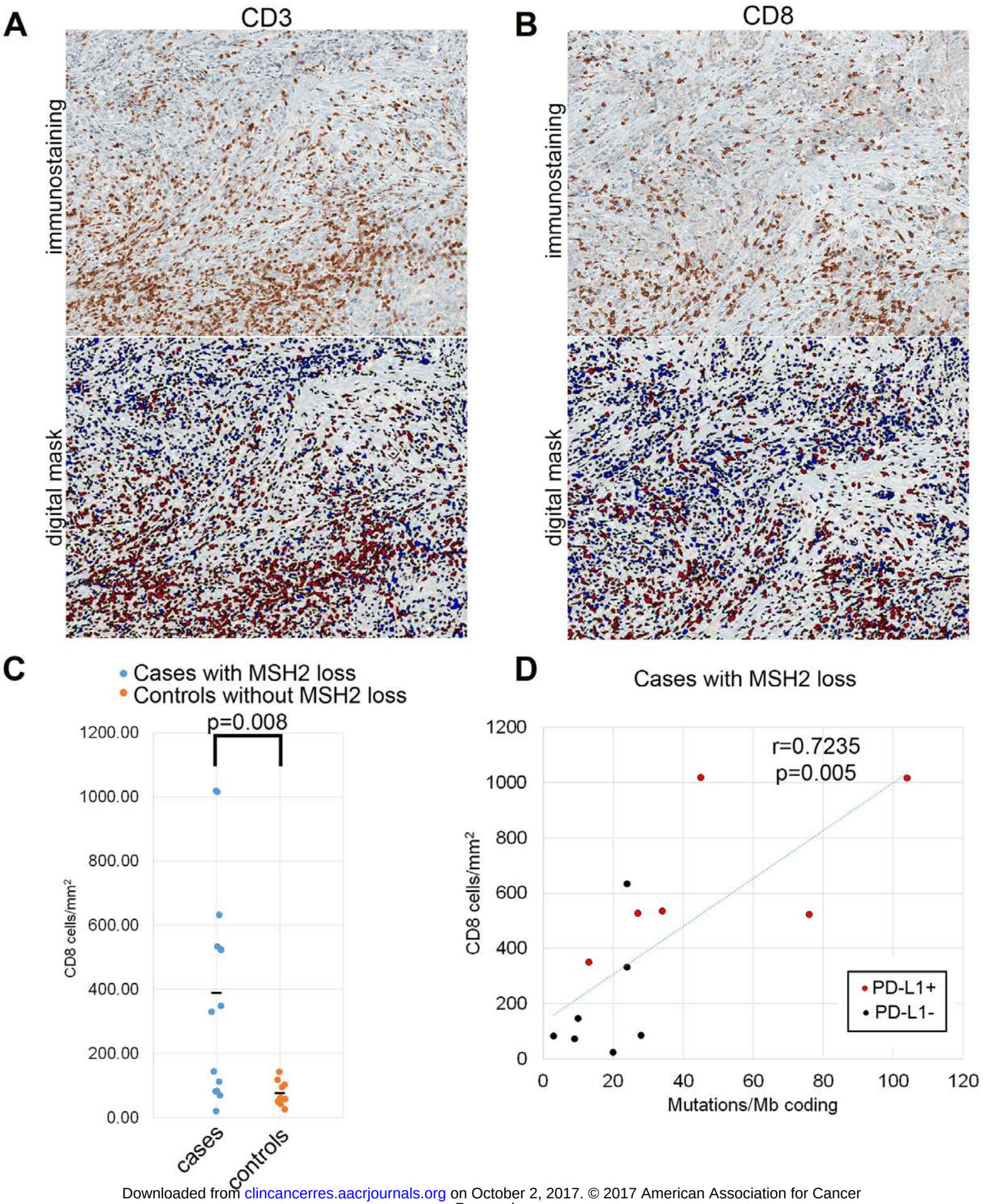
Figure 3



Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 4



Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.

Author Manuscript Published OnlineFirst on August 8, 2017; DOI: 10.1158/1078-0432.CCR-17-0955
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

AACR American Association for Cancer Research

Clinical Cancer Research

MSH2 Loss in Primary Prostate Cancer

Liana Guedes, Emmanuel S. Antonarakis, Michael T Schweizer, et al.

Clin Cancer Res Published OnlineFirst August 8, 2017.

Updated version	Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-17-0955
Supplementary Material	Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2017/08/08/1078-0432.CCR-17-0955.DC1
Author Manuscript	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2017 American Association for Cancer Research.